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IN VITRO EXPANSION OF DONOR-DERIVED CMV-SPECIFIC T LYMPHOCYTES FOR ADOPTIVE CELLULAR THERAPY: OPTIMISATION OF ANTIGEN PRESENTATION BY DENDRITIC CELLS, CHARACTERISATION OF THE CULTURE OUTPUT, AND ADAPTATION OF CULTURE CONDITIONS TO CMV-NAÏVE DONORS

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Declaration

The work presented in this thesis is completely the result of my own work

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Abstract

Cytomegalovirus infection adversely affects the survival of haemopoietic stem cell transplant recipients, despite antiviral chemotherapy. As an alternative treatment, adoptive cellular therapy can transfer specific immunity from donor to recipient, avoiding drug side effects. A range of methods for the isolation and/or enrichment of CMV-specific donor-derived T cells have been explored.

Our cell product comprises donor-derived CMV-specific T cells, enriched through co-culture with CMV antigen-presenting MoDC. These cells are currently assessed in two ongoing clinical studies.

To further optimise the co-culture procedure, the effects of various mediators of DC maturation on DC immunophenotype and their capacity for induction of antigen-specific T cell proliferation were evaluated. Whereas DC matured with a cytokine cocktail appeared to be the most mature as defined by surface maturation marker expression, only exposure to CD40L gave rise to the functionally most effective MoDC, leading to a reduction in the required co-culture time. Antigen loading and timing of cryopreservation steps were also optimised experimentally.

Knowing the composition of cultured cell products may result in improved protocols, and could help interpret data from clinical studies. The proportion of T cells binding HLA-CMV-peptide tetramers and the proportion of T cells secreting IFN-γ in response to restimulation increased form pre- to post-culture. However, in spite of the occurrence of massive T cell proliferation in response to CMV antigen, the majority of post-culture T cells neither bound tetramer nor secreted IFN-γ. There was partial overlap between tetramer-binding and IFN-γ-secreting CD8+ T cell populations, as indicated by TCR CDR3 spectratyping. IFN-γ secreting post-culture T cells were capable of further divisions, as verified through non-specific cytokine-induced proliferation.

Induction of cell products from CMV-naïve donors continues to remain a challenge. Altered culture conditions resulted in proliferation in some CMV seronegative donor cultures. Culture output did not bind tetramers, but IFN-γ secretion in response to CMV antigen, and skewing of post-culture spectratypes were demonstrated.

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Glossary

ag	Antigen
APC	Antigen Presenting Cell or Allophycoerythrin (context dependent)
BC	Constant Region-Beta
BLCL	B-Lymphoblastoid Cell Line
BM	Bone Marrow
bp	base pair
BSA	Bovine Serum Albumin
BV	Variable Region-Beta
CCR7	Chemokine (C-C Motif) Receptor 7, CD197
CD	Cluster of Differentiation
CD40L	CD40-Ligand
cDNA	complementary Deoxyribonucleic Acid
CDR	Complement Determining Region
CMV	Cytomegalovirus
CTL	Cytotoxic T Lymphocyte
CTLA	Cytotoxic T Lymphocyte-Associated Protein
df	degrees of freedom (Statistical)
DC	Dendritic Cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide triphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanine triphosphate
dTTP	Deoxytyrosine triphosphate

EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked ImmunoSPOT
ER	Endoplasmic Reticulum
FACS	Fluorescence-Activated Cell Sorting
FB	Fibroblast
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FITC	Fluorescin Isothiocyanate
gB	glycoprotein B
GCP	Good Clinical Practice
gH	glycoprotein H
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GMP	Good Manufacturing Practice
GvHD	Graft versus Host Disease
gy	gray (unit of absorbed dose of radiation)
HLA	Human Leukocyte Antigen
HVEM	Herpes Virus Entry Mediator
IE-1	Immediate Early Antigen-1
IFN	Interferon
IL	Interleukin
ILT	Immunoglobuling-Like Transcript
kb	kilobase
KLH	Keyhole Limpet Haemothianine
LC	Langerhans Cell
LDH	Lactase Dehydrogenase
LIGHT	LT-related inducible ligand that competes for glycoprotein D binding to herpes virus entry mediator on T cells
LPS	Lipopolysaccharide
Mabs	Monoclonal Antibodies
MACS	Magnetic-Activated Cell Sorting
MCM	Monocyte-Conditioned Medium
MCM-Mimic Cocktail	X-Vivo 20 medium with added IL-1, IL-6, TNF-α, and PGE_2
mDC	myeloid DC
MΦ	Macrophage
MFI	Mean Fluorescence Intensity
MGG stain	May-Grünwald Giemsa Stain
MHC	Major Histocompatibility Complex
MIEP	Major Immediate Early Promotor
MLR	Mixed Leukocyte Reaction
Mo	Monocyte
MoDC	Monocyte-Derived Dendritic Cell(s)
n	Sample Size
NA	Not Applicable
NAC	N-acetyl L-cysteine
ND	Not Done

neg	negative
NK	Natural Killer Cell
OKT-3	Anti-CD3 monoclonal antibody licensed for clinical use
PBHSC	Peripheral Blood Haemopoietic Stem Cell
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid DC
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PP	Phosphoprotein
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
SCT	Stem Cell Transplantation
SD	Standard Deviation
SEB	*Staphylococcus* Enterotoxin B
TAP	Transporter Associated with Antigen Processing
TCR	T Cell Receptor
Tet	Tetrameric Complex
TGF	Transforming Growth Factor
TH	T Helper
TNF	Tumour Necrosis Factor

CHAPTER 1

Introduction

1.1 Introduction

The topic of this dissertation is the generation of CMV-specific T cells for adoptive immunotherapy. The introduction will provide a background, briefly summarising the current understanding of CMV infection including mechanisms of viral persistence in the host, and the immunology of control of infection. The detrimental effect of CMV infection in haematopoietic stem cell recipients, and current strategies for treatment and management are outlined. The literature on the development of cellular immunotherapy for this patient group is reviewed in greater detail. Finally the aims of our research in this context are explained.

1.2 CMV

Herpes viruses cause persistent latent infections in humans. CMV, a member of this virus family, is a double-stranded linear DNA virus with a large genome of about 240 kb. The virion has 162 hexagonal capsomers and is surrounded by a lipid membrane. Prevalence of CMV infection in human populations ranges from 50 to 90%. After the acute phase of infection – which often remains undetected, but can be associated with symptoms resembling infectious mononucleosis - chronic or latent infection is generally free of clinical symptoms. In some infected cell types, latency can be interrupted by episodes of lytic infection. At such a time infectious particles can be found in the blood, urine, and are shed from all epithelial surfaces. Sporadic viral replication is kept in check by the host's immune system in healthy individuals. During the acute phase, CMV causes an infection of the salivary gland (review: Harari et al., 2004 [1]), whereas the latent reservoir is likely to be myeloid lineage cells in the bone marrow and blood, via which the virus is spread to every organ system of the body. CMV can enter various different cell types, but viral replication is restricted to a few. Monocytes, for example, are non-permissive for viral replication, whereas macrophages and DC further down the line of differentiation can become permissive under certain circumstances[2;3] (latency and reactivation reviewed: Sissons et al., 2002 [4]).

Although healthy infected individuals usually remain without symptoms, a very large portion of their adaptive immune system is dedicated to keeping control of viral replication. There tends to be a build-up T cells dedicated to the virus in the peripheral blood during the

course of the infection over time [5-7]. CMV infection can be transmitted laterally and vertically.

CMV infection can cause severe morbidity and mortality in situations of impaired immunity, e.g. during pregnancy, in AIDS, post-allogeneic transplantation, or during exposure to immunosuppressive drugs.

1.3 The Immune Response to Ongoing CMV Infection in Healthy Adults

In immunocompetent adults CMV infection is kept under control by a complex and multi-facetted host immune response. Antibodies against viral proteins, originating from activated B cells, can be detected in the serum of infected individuals. They contribute to the inactivation of infectious particles and thereby limit viral spread during episodes of lytic infection. Antiviral cytokines produced by the cells of the innate and adaptive parts of the immune system protect potential host cells from viral entry. Type 1 T cell responses have been found to be most closely associated with control of CMV infection, i.e. CMV-specific CD8-expressing CTL that specifically detect and destroy infected cells, as well as CD4+ T cells [8], which provide help to CTL and produce antiviral cytokines. Finally, NK cells destroy CMV infected cells with aberrant HLA-molecule expression, but their overall contribution to the control of CMV infection in humans is quite unclear, although following allogeneic SCT, the development of NK cell cytotoxicity was shown to be correlated to patients' recovery from CMV infection [9](reviewed: Ghandi et al., 2003 [10]).

1.3.1 Induction of Type 1 T Cell Responses to CMV

The induction of cytotoxic T cell responses requires presentation of antigen to CD4+ helper T cells and simultaneous presentation of antigen to CD8+ T cells on the same APC [11]. Antigen presentation involves ligation of TCRs with a peptide-HLA complex presented on an APC, stabilised by the specific binding of CD4 to the peptide-presenting HLA class II, or CD8 to HLA class I molecules, respectively (Figure 1.1). CD3 is directly associated with the TCR for signal transduction. The length of time of the interaction and affinity of binding and the resulting strength of signalling to the T cell then depend partly on the amount and type of co-stimulation. Co-stimulation is mediated by the ligation of molecules

in the T cell plasma membrane and their ligands on the APC in the immediate vicinity of the TCR-peptide-HLA complex. These molecules are mostly members of the TNF and CD28 superfamilies. Professional APC have large amounts of ligands for T cell co-stimulatory surface molecules as well as accessory molecules that will facilitate binding of T cells to APC. For example, T cell CD154 is a ligand for CD40. CD40 is constitutively expressed on professional APC. Cross-linking of CD40 mediates DC maturation and hence upregulation of CD80 and CD86, which are ligands for T cell CD28 and for the inducible negative regulator CTLA-4.

The nature of the resulting T cell response is a consequence of the combination of the signals received directly through TCR ligation with the HLA-peptide, the sequence and amount of positive and negative co-stimulation, and the external cytokine environment. Both, T cells and APC contribute to the nature of the T cell response by their cytokine secretion patterns. For example, IL-1 secreted by APC during the antigen presentation process induces T cell IL-2 and IL-2 receptor upregulation. T cell activation is maintained through an autocrine loop mediated by IL-2. Primed CD4 helper cells can then secrete type 1 cytokines such as IL-2 and IFN-γ, which serve as secondary signals in the activation of primed CD8-CTL (Figure 1.1). Alternatively, type 2 cytokines, such as IL-4 and IL-5, are secreted, and act on primed B cells. Once activated, CD4 T cells become committed to either type 1 or type 2 cytokine production, thereby polarising the type of adaptive immune response to specific antigens. The route taken by T cells is dependent on the type of antigen and the activation status of the APC. Activation of an inappropriate kind of T cell response can result in persistence of the pathogen against which it was directed, and collateral damage induced by chronically maintained ineffective immune activation. Cytokines secreted by activated APC or T cells also serve to recruit effector cells of the innate immune system to sites of infection.

Initial priming of type 1 T cell responses takes place in the T cell areas of the lymph nodes, to which activated, antigen-loaded DC migrate. Antigen-specific T cells proliferate and disperse from lymph nodes to sites of infection in the efferent blood stream. In type 1 responses, CD4+ antigen-specific T cells can be detected in the blood first, followed by an overlapping surge of antigen-specific CD8+ T cells. As the antigen load decreases, expression of negative co-stimulatory molecules increases, resulting in secretion of

inhibitory cytokines, such as IL-10 that help terminate T cell responses. After clearance of the antigen, the vast majority of antigen-specific T cells apoptose. A small percentage of antigen-specific T cells, both CD4 and CD8 expressing, remains as memory T cells: as central memory T cells (CD45RO+, CD27+, CD28+, CCR7+), which will home to secondary lymphoid organs ready to induce memory responses upon re-challenge with the same antigen, or as effector memory T cells (CD45RO+, CD27+, CD28+, CCR7-) that circulate in the blood stream. CD45RA+, CD27-, CD28-, CCR7- is a third phenotype of circulating T cells found after repeated exposure to the same antigen, especially in persistent viral infections such as CMV. Even though this phenotype had previously been thought to be that of terminally differentiated T cells, these T cell can undergo further rounds of proliferation and re-express RO in the presence of TH-1 cytokines [12].

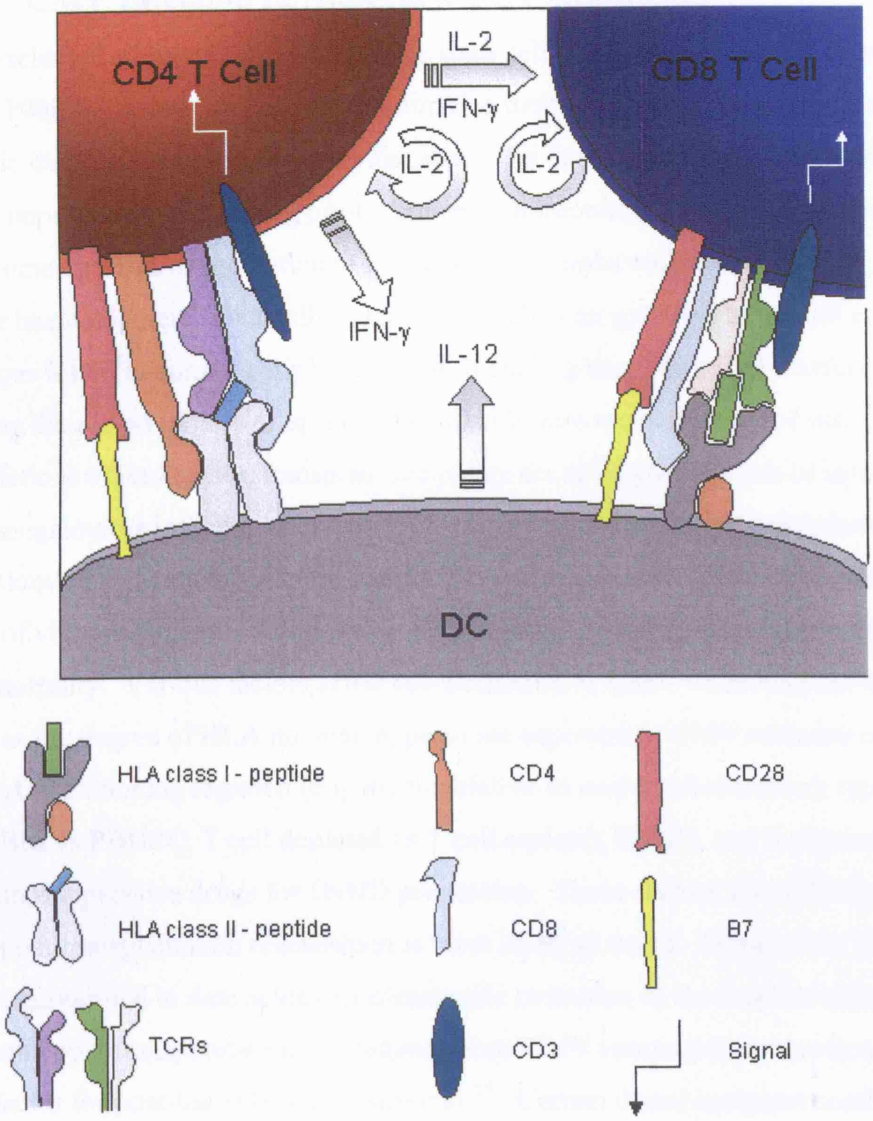

Figure 1.1 Diagram of the Induction of TH1 Responses

DC present peptides to TCRs on CD4 or CD8 expressing T lymphocytes via HLA molecules. CD4 molecules enable TCR recognition of peptides presented on HLA class II molecules by APC, whereas CD8 molecules restrict TCR recognition to peptides presented on HLA class I molecules. Ligation of T cell CD28 to B7 molecules on the APC can provide the second signal required for signal transduction through CD3 resulting in cytokine production by T cells. For a TH1 response, CD4 cells secrete type I cytokines such as IFN-γ and IL-2, which provide help for the activation of CD8 T cells interacting with the same DC. IFN-γ also induces IL-12 secretion by DC, which enhances T cell activation.

1.4 CMV Infection in Allogeneic SCT Recipients

HLA-selected allogeneic haematopoietic stem cell transplantations have been successful since 1968 [13], serving as a potentially curative treatment for haematological malignancies, genetic disorders, and autoimmune diseases. The destruction of the host's diseased haematopoietic system during pre-transplant conditioning with e.g. myeloablative chemotherapy and/or irradiation is followed by its replacement with healthy, HLA-matched donor haematopoietic stem cells, which eventually can give rise to the full range of cell lineages found in normal peripheral blood, including the cells of the immune system. During the period of time after transplantation before reconstitution of sufficient populations of leukocytes, transplant recipients are at heightened risk of infections. Cytomegalovirus infection or re-activation is one of the main post-transplantation infectious complications, affecting about 50% of all patients. If left untreated, roughly one third of viraemic patients will develop overwhelming infection, causing severe morbidity and mortality. Various factors affect the likelihood of CMV viraemia post-transplantation, such as the degree of HLA mismatch, previous exposure to CMV of donor or recipient, type of conditioning regimen (e.g. myeloablative vs non-myeloablative), type of transplant (e.g. BM vs PBHSC, T cell depleted vs T cell replete), GvHD, and treatment with immunosuppressive drugs for GvHD prevention. These factors also affect at what point in time post-transplantation reactivation is most likely to occur. None of the conditioning regimens applied to date achieve a measurable reduction in the total incidence of CMV viraemia post-transplantation [14]. Patient/donor CMV seropositivity remains an important risk factor for post-transplantation survival [15]. Certain donor-recipient combinations bear an increased risk of CMV disease. Recipients of transplants from unrelated donors and/or of transplants with a greater degree of tissue mismatch bear a higher risk. CMV infection frequently reactivates in transplant recipients who have already been infected before transplantation. CMV infection can also be transferred from a CMV seropositive donor to the recipient along with the graft. A CMV positive recipient of a CMV negative graft bears the highest risk due to the lack of any immunological memory transferred from the donor. Prophylactic treatment of patients with antiviral drugs such as Gancyclovir and Foscarnet, has greatly reduced the incidence of early onset CMV disease. However, this reduction in early CMV disease has not been associated with an increase in overall long-term survival.

This may be attributed to adverse side effects of the antiviral chemotherapy, such as bone marrow toxicity, which can result in neutropaenia (in the case of Gancyclovir), and with failure to establish a long-term protective immune response against CMV, putting patients at risk of late onset CMV infections and other infections. The use of Foscarnet, which has less bone marrow toxicity, is limited by its renal toxicity. The availability of improved techniques, e.g. qPCR, for low-level CMV viraemia screening has led to better targeting of antiviral chemotherapy. In pre-emptive protocols antiviral chemotherapy is targeted to patients most likely to benefit, at the time of viral DNA detection in the peripheral blood, before development of any symptoms, thus reducing unnecessary exposure to drug side effects. As a result CMV disease early after transplantation has become a rare event. However, the early clearance of viraemia by chemotherapy is associated with failure to establish effective adaptive cellular anti-CMV immune responses, which may explain why treatment at this early stage post-transplantation may lead to recurrent viraemia, and puts patients at risk of late-onset CMV disease [15;16].

Consequently, the availability of alternative treatments with fewer side effects would be advantageous. There may be scope for development of drugs with fewer side effects. Further optimisation of conditioning regimens and post-transplantation medication to strike the balance between minimisation of risk of infections, prevention of GvHD, maintenance of maximum graft-anti-tumour effect may be possible. However, the more novel approach of cellular immunotherapy for CMV has already shown some effectiveness in initial clinical trials.

1.5 CMV Antigens

The CMV antigens exposed to the immune system vary during the course of the infectious cycle. Viral genes are expressed in 3 distinct stages: immediate early, early, and late. Early detection of infected cells by the immune system and their destruction can prevent the release of further infectious particles. The CMV phosphoproteins are tegument proteins which are expressed late in the cycle. Although expressed late, the lower matrix pp65 is 'visible' to the immune system on the surface of infected cells during the process of virion entry, before the start of any viral gene expression [17]. CMV-CTL targeting pp65 can recognise and delete infected cells before the onset of viral replication. This makes pp65 a

very effective target of the cellular immune response for limiting infection. Another such target is IE-1. The IE-proteins are the first viral genes expressed after infection of a cell. They act as non-specific strong activators of gene expression, conditioning host cells for viral gene expression and replication. Targeting IE-1 can prevent completion of the viral productive cycle. Other antigens to which strong T cell reactivity occurs in healthy infected donors include pp50, gB (involved in viral attachment), and IE-2. There are also weaker T cell responses against other antigens such as pp28, pp150, and gH (involved in viral attachment) [18;19]. Around 60% of total T cell responses are directed against the two antigens pp65 and IE-1 [19]. gB is a predominant target for neutralising antibodies.

1.6 How CMV Persists by Manipulating the Host's Immune Response

Why does the immune system not succeed in eradicating the virus from the body? CMV, despite expressing very immunogenic proteins, has evolved a whole host of mechanisms to evade the immune system.

The co-evolution of CMV and the immune system of its human host over a long period of time has resulted in a delicate balance resulting in persistent, but controlled infection, causing no measurable symptoms to the host under normal circumstances. CMV has evolved a whole range of mechanisms by which it succeeds in persisting in its host, whereas the host's immune system dedicates comparatively huge resources to keep the infection under control, usually successfully.

Here are some examples of viral mechanisms for "evading" the host immune response:

First of all, CMV reduces exposure to the host's defences through limiting levels of viral gene expression during latency, whereas viral replication occurs mainly in immunoprivileged sites.

CMV has a host of gene products that seem to be dedicated exclusively to interfering with the host immune response. Many of these viral proteins affect antigen processing and presentation by host cells, such as the interference with TAP-mediated transport of peptides into the ER, the trapping HLA I molecules in the ER, and the shortening the half-life of HLA component chains. The resulting low-level of HLA molecules in the plasma membrane would normally trigger NK cell-mediated lysis, but this can be prevented by virally-encoded HLA class I homologues (reviewed by Hengel *et al.*[20]). Viral gene

products also down-regulate HLA class II molecules on the host cell plasma membrane [21]. The virally induced induction of host cell cytokine expression, such as TGF-β, can stimulate viral replication while at the same time inhibiting T cell proliferation. Molecular mimicry of chemokine receptors is employed by CMV to mop up host chemokines. CMV also encodes a homologue of the predominately immunosuppressive cytokine IL-10. Furthermore, CMV can inhibit maturation of infected DC, thereby affecting their ability to prime an effective T cell response [22]

These are examples of strategies employed by CMV for persistence and dissemination. The viral gene products vary in their effects on different host cell types. For example, infected fibroblasts have reduced numbers of HLA class 1 molecules presenting viral antigens, but the mechanisms for reduced HLA expression do not work in macrophages.

To summarise, CMV infection greatly shapes the host's adaptive immune response, but usually is not a cause of measurable disadvantage to the human host, except perhaps for its implication in old age immunosenescence [23,5,6]. When the host's immune system is perturbed, e.g. after HSCT, the elaborate persistence mechanism of the virus can result in uncontrolled infection, leading to CMV disease. The side effects of inappropriate immune responses can then result in even greater damage to the host.

1.7 DC

In the context of CMV infection DC play a role as host cells, but they are also key to the control of infection through induction of appropriate T cell responses.

DC are a heterogeneous group of CD45+ leukocytes, that are lineage negative for CD3, CD19, CD14, CD15, CD56. They are characterized by expression of high levels of MHC class II molecules, and have the ability to induce primary T cell responses after activation and terminal maturation.

DC form the major link between the innate and adaptive immune system. In the innate system their role is to recruit other DC, NK cells, neutrophils, and other effector cells of the innate immune response to sites of inflammation where non-self antigen is encountered. Antimicrobial cytokines, such as IFNs, are also produced by DC themselves.

The other major role of DC is as initiators and coordinators of the adaptive immune response: Upon encounter of non-self antigen in a pro-inflammatory microenvironmental setting DC will undergo maturation, enabling them to translocate to the lymph-nodes where they may initiate primary T cell responses. Beyond this, DC also influence T cell homing to tissues, and the type of T cell response induced to a particular antigen, i.e. TH-1 or TH-2 polarisation by cytokines they produce.

Another function of DC is to induce tolerance to self-antigens by two mechanisms: a) by deletion of self-reactive T cells, b) by the induction of regulatory T cells.

In the steady state iDC continuously sample their environment and process self-antigen (e.g. apoptosing cells). This way DC become loaded with antigen, which can lead to a degree of activation in the steady state. Antigen loaded DC then translocate to the lymph-nodes to present self-antigen to T cells, leading to partial activation and limited proliferation of the reactive T cells, but with a second signal missing, either T cell anergy, or apoptosis is induced. Mature DC that induce T cell responses to non-self antigens will also always present self antigens at the same time. For this reason it is vital that T cell anergy is induced before encounter of foreign antigen, avoiding the induction autoreactivity.

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The origin of DC can be obscure. Both myeloid and lymphoid precursors can give rise to DC [24]. However, the progeny of myeloid and lymphoid DC precursors is difficult to identify in tissues. Human blood contains two DC subsets: myeloid and plasmacytoid DC, as defined by morphology and surface marker expression. pDC bear structural resemblance to plasma cells and are CD4+, whereas mDC exress myeloid markers and display monocytic morphology. However it is likely that both mDC and pDC can originate from lymphoid or myeloid precursors[25]. pDC and mDC have distinct functional bias. pDC play a great part in antiviral immunity through Interferon production, they are thought to preferentially induce TH2 responses, and are implicated in the induction of tolerance. mDC, on the other hand have been implicated in TH1 responses (reviewed by Arpinati *et al.*[26]). Known characteristic differences between mDC and pDC are displayed in Table 1.1.

DC are the most potent APCs and probably the only cell type capable of inducing primary T cell responses. To be able to prime CD8 responses in situations where DC are not themselves infected with invading organisms or viruses, they need to be able to cross-present exogenous peptides on MHC class I molecules[27]. Several mechanisms for cross-presentation are known: macropinocytosis delivering soluble antigens into the cytosol[28], the cross-over of peptides generated in the endocytic antigen processing pathway to the cytoplasm for presentation on HLA class I molecules to CD8 T cells (a mechanism not understood to date)[29], exchange of peptides on mature MHC class I molecules (probably rare[30]), presentation of peptide-loaded HLA class I molecules from e.g. apoptotic cells[31], or chaperones delivering exogenous peptides into the ER via the cell surface (reviewed by Li *et al.*[32])
Some DC can also secrete exosomes (membrane vesicles comprising antigen-loaded HLA molecules and accessory molecules), which can integrate into the membranes of other DC allowing them to present the same antigen[33].

In vivo, different stages of DC differentiation/activation are intricately associated with their location in certain tissue sites and organ systems within the body. There are LC in the skin, interstitial DC in all organ systems, interdigitating DC in lymphnodes, veiled DC in afferent blood vessels, blood DC, mucosal DC, and MoDC. In fact, it remains unclear at present to what extent DC sub-types represent truly separate lineages, as opposed to how far DC with different properties and phenotypes are a result of their activatory or regulatory history, and thereby just reflecting their immense plasticity in response to different stimuli. For example, mDC precursors in the in the blood can differentiate into tissue dwelling iDC with completely different properties and morphology. These in turn end up as antigen-loaded DC, either terminally mature, or steady state, which leads them to change their location, morphology and functional properties once again.

As they differentiate, iDC (e.g. LC) change their gene expression profile from one associated with mainly antigen uptake, to one associated with antigen processing, to finally one of effective antigen presentation to T cells followed by apoptosis. Concurrent with this are changes in the cytoskeleton, motility, and organs/tissues targeted. To enable these

changes, DC at various stages of differentiation have to be able to modulate expression of gene products associated with antigen uptake (e.g. toll-like receptors, LPS receptors, molecules associated with pinocytosis) cytokines and their receptors (e.g. induction of IL-12, IL-6, IL-1, TNF-α, IFN-γ, or IL-10 expression after activation), surface molecules associated with antigen-presentation (MHC molecules, accessory molecules, as discussed in greater detail in chapter 3), and expression of adhesion molecules and chemokine receptors (e.g. the change from CCR6 expression, targeting DC to the dermis, to CCR7 for chemotaxis to the lymphnodes, induction of DC-SIGN involved in rolling along endothelium).

Control of DC differentiation at the molecular level involves a multitude of NF-κB transcription factors enabling a rapid reaction to a variety of stimuli. Signalling occurs mainly through Map-kinase mediated pathways, enabling selective recruitment of the various NF-κB transcription factors and resulting in changes in gene expression (Reviewed by Steinman [34]).

Only mature DC can initiate primary T cell responses. Mature DC are terminally differentiated, i.e. they can neither differentiate any further before apoptosis, nor are they able to revert back to a previously adopted phenotype. It appears, however, that even when terminally differentiated, DC can vary in their phenotype. DC have the potential to activate primary T cells, provided they encounter certain signal sequences during the process of their maturation. However, if DC are subjected to different signals they may become terminally differentiated without acquiring the ability to induce primary T cell responses. It follows that not all terminally mature DC are capable of inducing primary T cell responses. This issue is discussed in more detail in chapter 3. As immature DC differentiate into mature DC, they undergo intermediary stages differentiation, that may be reversible. DC in such intermediary stages of differentiation are also described as 'activated' if differentiation occurs as a result of exposure to pro-inflammatory signals.

Table 1.1 DC Subsets

	Myeloid Dendritic Cells (DC1)	Lymphoid Dendritic Cells (DC2)
Surface markers	CD13, CD33, CD11c, ILT-1	CD4, CD64, IL-3 Receptor α Chain
Morphology	Myeloid	Plasmacytoid
Cytokines produced in mature state	Interleukin-12	Type 1 Interferons
T cell response induced	TH1	TH2, Treg, TH1
Preferential Location	Most Peripheral Tissues	Secondary Lymphoid Organs
Migration/homing	Migration in response to inflammatory cytokines, homing to lymphnodes	No migration in response to inflammatory cytokines, homing to lymphnodes
Chemokines produced	Homeostatic Chemokines	Pro-Inflammatory Chemokines
Toll-Like Receptors	TLRs 1,2,4,5,8	TLRs 7,9
Response to microbial products	LPS	CpG-Oligodeoxynucleotides
Antigen Uptake	Phagocytosis, pinocytosis, macropinocytosis	Low antigen uptake

ILT-1 = Immunoglobuling-like transcript-1
CD = cluster of differentiation
TH = T helper
Treg = regulatory T cell
TLR = Toll-like Receptors
LPS = Lypopolysaccharide
CpG = Cytosine-phosphate-Guanine

1.7.1 CD83

CD83 expressed on the DC surface is widely regarded as a marker of their mature/activated state. CD83 is not unique to DC; T cells, Mo, Mϕ and endothelial cells also express species of CD83, but only mature DC express CD83 stably on their surface [35]. In its transmembrane form, CD83 is a 45kd glycoprotein, and is related to B7. Pre-formed intracellular CD83 can be rapidly expressed on the cell surface, in a process requiring post-translational glycosylation [35]. Surface CD83 on mature DC promotes proliferation and

survival of naïve CD8+ T cells that have already encountered the first and second signal, by binding to an as yet uncharacterised T cell ligand forming part of the immunological synapse [36]. CD83 also has several splice variants lacking the transmembrane and intracellular domains in DC and other cells[37]. Such soluble CD83 species are present at low levels in normal human serum. In its soluble form CD83 performs regulatory functions, blocking antigen presentation to T cells and inhibiting DC maturation. Shedding of surface CD83 may be an additional mechanism for the generation of soluble CD83. To summarise, serum CD83 contributes to preventing inappropriate T cell activation, and the production of soluble CD83 is tightly regulated. In response to danger signals, DC can rapidly switch to expression of surface CD83, required for activation of naïve T cell and their survival.

1.7.2 DC and CMV

In healthy carriers, CMV is latent in a small percentage of Mo, Mϕ, DC, and their myeloid precursors. CMV can also directly infect these cells. Myeloid cells latently infected with CMV become permissive to lytic infection during their differentiation, a process completely dependent on host factors, and not requiring any viral gene expression. In this way normal differentiation of Mo into Mϕ, or DC maturation enables CMV to reactivate periodically.

IE gene expression is controlled by MIEP, which can bind cellular transcription factors when in an open chromatin formation, but not in the closed chromatin formation encountered in less differentiated myeloid cells. A balance of cellular regulatory and activatory factors binding upstream of MIEP maintains latency in non-permissive cell types. In permissive cell types the binding fewer cellular repressors alters the chromatin structure around MIEP and enables IE gene expression.

DC, as hosts of latent and lytic CMV infection may be the responsible for the extremely high frequencies of CMV-specific CTL seen in the blood of healthy infected individuals, due to their highly immunogenic nature. The virus, however, also interferes with DC maturation and antigen presentation. DC directly exposed to virions express reduced numbers of HLA molecules (e.g.[38]) and co-stimulatory molecules on their surface. They induce less allogeneic T cell proliferation than DC not exposed to CMV in *in vitro* assays

[39,40]. CMV infection can also alter the DC cytoskeleton, resulting in the loss of dendrites that are required for effective antigen presentation to T cells [40].
It is possible that DC pulsed *in vitro* with CMV lysate may still be subject to some of these inhibitory functions.

1.8 Adoptive Cellular Immunotherapy

1.8.1 Clinical Studies: Adoptive CMV-Specific Cellular Therapy

The rationale behind the idea of adoptive cellular immunotherapy was the recognition of the protective role of CMV-specific T cells after transplantation. Adoptive transfers of CMV-specific CTL resulted in control of CMV infections in irradiated mice [41;42]. Furthermore, in humans levels of CMV-specific CTLs post BMT transplantation were shown to be associated with protection from CMV disease [43]. In 1995, Stanley Riddell and co-workers [44] showed for the first time that that adoptive transfers of CMV-specific CTL following bone marrow transplantations were safe and feasible. In this first clinical study, donor-derived CD8+ T cell clones were expanded after stimulation with autologous CMV-infected fibroblasts and infused at doses ranging from $3.3x10^7/m^2$ to $1x10^9/m^2$ in a prophylactic protocol, 28-42 days after transplantation. There were none to only mild side effects in 14/14 patients, and in 11 patients who were deficient in CMV-specific cytotoxic activity before adoptive immunotherapy, such activity was detected by *in vitro* assays after infusions of CMV-specific CTL clones. The clones persisted for at least 12 weeks, but decreased over time if CMV-specific CD4-T cell responses were lacking. This indicated the importance of CMV-specific CD4 T cells for the maintenance of CMV-CTL.
Three further clinical studies of adoptive transfers of CMV-specific T cells have since been published. Two approaches avoided cultures containing virions by stimulating T cells with APC pulsed with a crude CMV antigen. Einsele *et al.* [45] cultured lysate-pulsed donor PBMC for several weeks, followed by a period of non-specific expansion. The resulting T cell product contained almost exclusively CD4+ T cells. Given to patients not responding to antiviral chemotherapy, these cells conferred protective immunity in 6/7 patients, and transient reductions in viral loads in all patients.

Our group expanded both CD4 and CD8 CMV specific T cell lines *in vitro* from CMV seropositive donor blood using CMV lysate-pulsed MoDC as APC. 12 of 14 patients treated pre-emptively after detection of CMV DNA in their blood had no further episodes of CMV viraemia [46].

In the most recently published study CMV-specific T cells were isolated from donor aphaeresis products by labelling with HLA-CMVpeptide tetramer followed by magnetic-activated selection, and infused without further modifications [47]. This treatment lead to a reduction in viral load in 9/9 patients, CMV-specific CD8 T cells were detected in all patients by day 10. Only 2/9 patients required treatment with Gancyclovir. The infection was controlled in 8/9 patients, including in one patient with persistent CMV viraemia. Interestingly, T cell function of CMV-specific CD8+ T cells, as measured by IFN-γ-secretion, was shown to decrease over time post-infusion. Clinical studies are summarised in table 1.2.

Table 1.2 Clinical Trials

Paper	Cell product and dosing	Patients treated	Testing of cell product	Patient monitoring	Result
Walter *et al.*, 1995 [44]	Adoptive transfer of CD8+ CMV-specific T cell clones (4 infusions), escalating doses from 33 million to 1billion cells/m^2	HLA-matched allo BMT recipients from CMV+ donors, 30-40 days after transplant. Did not receive antiviral drugs	CMV-specific cytotoxicity	post-infusion T cell proliferation and cytotoxicity. TCR BV clonotypic PCR. Monitoring for CMV	CMV CTL reconstitution in 14/14 patients. Persistence of CTL for min. 12 weeks. No viraemia or CMV disease in any patients. CD4 needed for CD8 maintenance
Einsele *et al.*, 2002 [45]	PBMC stimulated with CMV-lysate (up to 28days), restim. with autologous feeder PBMC from day 10 onwards. Dose: 10^7/m^2	Recipients of transplants from CMV+ donors, experiencing persistent recurrence of CMV infection not responding to anti-viral drugs.	alloreactivity testing by T proliferation assay, Intracellular IFN-γ staining, tetramer staining	T cell proliferation, CMV DNA load, tetramer and IFN-γ staining	Reduction in viral load in 7/7 patients, transient effect in 2/7 patients (immune-suppressive treatment), successful in 6/7 patients
Peggs *et al.*, 2003 [46]	2-week co-cultures of CMV lysate-pulsed MoDC and autologous T cells. One re-stimulation with antigen-pulsed MoDC, IL-2 from day 10 onwards. Dose: $1x10^5$ cells/kg	Recipients of a transplant from CMV+ donors, adoptive cellular therapy after detection of CMV DNA by qPCR.	Proliferation assays, LDH-release cytotoxicity assays	T cell reconstitution after treatment with CMV-CTL. Tetramer staining. TCR CDR3 spectratyping. CMV qPCR	8/16 patients cleared viral load without drugs. 12/14 had no further episodes of CMV reactivation
Cobbold *et al.*, 2005 [47]	Adoptive transfers of unmanipulated HLA-CMVpeptide-tetramer+ CD8+ T cells isolated with magnetic beads	Recipients of transplants from CMV+ donors: 7 patients treated after initial episode of CMV reactivation, 2 patients after persistent infection.	Tetramer staining, cytotoxicity assays, non-specific expansion	Tetramer staining, monitoring of viral load, TCR BV clonotypic PCR. IFN-γ secretion assay	CMV-specific T cells detected in 9/9 patients by day 10, reduction in viraemia in 9/9, control of infection in 8/9 patients (incl. One with persistent viraemia). Antiviral treatment required in 2/9 patients.

1.8.2 Generation of CMV-Specific Cellular Products

Since the first proof of principle, several groups have been working on modified methods for the isolation and/ or expansion CMV specific T cells for immunotherapy: to improve compliance with GMP and GCP, to simplify the method, to obtain certain T cell subsets, and to obtain more defined and reproducible cell products. A summary of publications on the generation and/or isolation of CMV-specific T cells is shown in table 1.3.

Cell products can be obtained by selection of CMV-specific T cells, by selective expansion of CMV-specific T cells in culture, or by a combination of both (figure 1.2).

For *in vitro* cultures, the choice of the type of APC and choice of antigens greatly affects the resulting T cell products. There are regulatory and safety concerns about using infected fibroblasts as APC. Professional APC, such as monocytes, DC, and less commonly B cells and BLCL, pulsed with antigens can replace infected cells as inducers of T cell responses. Both, monocytes and DC, present short peptides efficiently to CD8+ T cells without the requirement for endogenous processing. Larger antigens such as single proteins or crude lysates require intracellular processing. Monocytes will process protein antigens via the exogenous pathway and present the resulting peptides on HLA class II molecules to CD4 T cells. As well as presenting antigen to CD4 T cells more efficiently than Mo, DC have the capacity to cross-present exogenous antigen to CD8+ T cell on HLA class I molecules. Several groups used DC pulsed with single immunodominant peptides as inducers of CMV-specific T cell activation [48-50]. DC or PBMC pulsed with CMV lysate have both been used to generate T cells used in clinical studies [45;46;51]. Monocytes can be pulsed by adding antigen directly to PBMC avoiding isolation steps [48;52]. Pulsing PBMC with both peptide and protein together can induce CD4 and CD8 T cell activation, overcoming some of the limitations of Mo compared to DC as APC [52]. BLCL can be used to simultaneously generate CMV and EBV-specific T cells [53], and a clinical trial is underway [54].

Our efforts to optimise antigen presentation using MoDC are described in chapter 3.

Table 1.3 Generation/Isolation of CMV-Specific T Cells for Immunotherapy

Paper	Method	Result	Tests Applied	Comments
Sun *et al.*, 1999 [53]	Generation of CMV and EBV-specific CTL using pp65-transduced EBV-transformed BLCL as APC	Specific lysis of autologous CMV and EBV targets in 4 donors, mediated by CD8 CTL	Chromium release cytotoxicity assays, BLCL, FB as targets, auto and allo, uninf. and inf.	GMP: Safety of BLCL and retroviral transduction? FBS media
Vannucchi *et al.*, 2001 [55]	Co-cultures of MoDC pulsed with single pp65 peptide and T cells for 3 weeks, weekly restimulations and IL-2	Increasing numbers of IFN-γ secreting T cells during course of culture, Cytotoxicity mediated by CD8+	IFN-γ secretion assay, Cytotoxicity against peptide T2 cells (by flow cytometry)	
Verfuerth *et al.*, 2001 [56]	Expansion of CMV-specific T cell lines using CMV-lysate-pulsed MoDC	Expansion of CD4 and CD8 T cell lines from CMV seropositive donors	Proliferation, cytotoxicity (LDH-release assay), TCR CDR3 Spectratyping	How much alloreactivity?
Vaz-Santiago *et al.*, 2001 [57]	Stimulation of PBMC with chimeric protein: IE-1-pp65	Activation of ag-specific CD4 T cells. Proliferation of pp65 peptide - specific CTL. Killing of chimeric protein-pulsed targets by IE-1-specific T cell clones	Intracellular cytokine staining (IFN-γ), HLA-tetramer staining, Chromium release cytotoxicity assay (CD4+ and CD8+ mediated cytotoxicity), Cytokine ELISA	Not looked separately at pp65 and IE-1 induction by chimeric protein
Kleihauer et al., 2001 [50]	CMV-specific T cell induction with single Peptide-pulsed DC. Weekly restim + IL-2	Successful induction in 6/14 seropositive, 2/11CMV seronegative donors (6x restim)	Chromium release cytotoxicity assays with peptide-pulsed T2 or infected FB as targets. Tetramer staining	Media contained FCS. No tetramer staining for CMV neg donors demonstrated.
Szmania *et al.*, 2001 [49]	Co-cultures of DC pulsed with a single peptide and T cells (PBL or CD8+) for 21 days, re-stims. with peptide-LCLs or B cells. Tetramer-based isolation, expansion of tet+ cells (IL-2, IL-7, OCT3) for 10 days	17/19 seropositive cultures successful, 2/10 seronegative cultures successful (only in presence of CD4 T cells)	Cytotoxicity assay (Chromium release), tetramer staining, Intracellular cytokine staining (Tc1 phenotype), immunophenotype, spectratyping	Limited to single peptide restricted by HLA A2. CMV seronegative culture: Cytotoxicity data shown, but no tetramer staining demonstrated.
Bissinger *et al.*, 2002 [58]	Isolation of pp65 single peptide-specific T cells from PBMC by IFN-γ-capture MACS. Expansion of peptide-specific T cells with IL-2 and allogeneic irradiated feeder cells	Starting from 100ml blood: Expansion from 1-5x10^4 T cells to median 1.6x10^9. Up to 4 weeks to obtain 1x10^7 cells.	70-99% purity by tetramer staining and by Intracellular IFN-γ stain. Perforin+. Cytotoxicity: lysis of peptide-pulsed T2 cells. Cytotoxicity of CMV-infected FB targets.	Method adaptable to CMV-protein antigen?
Einsele *et al.*, 2002 [52]	Preferential induction of CMV specific CD8 or CD4 T cells using DC or Mo pulsed with either a single peptide- or with CMV protein.	Protein pulsed PBMC induced CMV-specific CD4 T cell lines. HLA class 1 restricted peptide-pulsed MoDC induced CD8 CTL. More re-stimulations were required for CMV neg donors (less than 20% success)	Elispot: after several re-stimulations 90% (seropos) 10% (seroneg) increase in peptide-specific T cells	No figures presented
Foster *et al.*, 2003 [48]	Single peptide-pulsed DC+PBMC, restim. day 7. Non-specific expansion of CTL with IL-2 from day 14 onwards	Successful expansion in 6/8 seropos. Donors, 0/3 seronegative.	HLA-tetramer stain, cytotoxicity assays (Chromium release)	Significant non-specific T cell expansion
Foster *et al.*, 2004 [59]	Enrichment: TNF-α-matured DC, pulsed with single CMV peptide, co-cultured with PBMC, twice weekly restimulations. IL-2 from day 7 onwards. Expansion: Restimulation with irradiated allo PBMC, BLCL +PHA or okt3. 50U/ml IL-2 from day 5. Further expansion: Transfer to suspension cultures	No difference between suspension and static culture. Maintenance of CMV specificity	Metabolic rates: lactate production, glucose consumption. Specific lysis of peptide-pulsed T2, 95% tetramer+	Minimum 6 week culture. Safety: allogeneic feeder cells, open culture system
Tazume *et al.*, 2004 [60]	APC: CD14+-derived DC, pulsed with CMV lysate, matured with TNF-α and LPS, weekly re-stims+IL-2	Success in 5/6 seropos patients, 4/6 seroneg	Cytokine ELISA and Intracellular IFN-γ staining, proliferation (pre and post culture), cytotoxicity (Chromium), killing mechanism: perforin (in seropos), Fas-ligand (in seroneg)	DC maturation not confirmed. No proliferation data for CMV seronegative donors. CMVneg cytotoxicity after 16-h assay. Little experimental data shown.
Ramadan *et al.*, 2004 [61]	Comparison of fast and standard MoDC. Matured, MoDC pulsed with CMV lysate, or *A. fumigatus* crude antigen, or infected with Adeno-pp65 as inducers of CMV- and Aspergillus-specific T cell lines. Aspergillus cultures +IL-12. Weekly re-stims (3 total)	Fast DC as good as standard DC at inducing ag-specific T cell expansion	T cell proliferation, Cytotoxicity assays (Chromium release)	How many experiments?
Rauser *et al.*, 2004 [62]	PBMC pulsed with peptide and lysate, CliniMacs selection of IFN-gamma-secreting T cells. 7-day non-specific expansion with feeder cells and IL-2.	Combination of peptide and lysate works best for stimulation of CD4 and CD8	Tetramer staining, Intracellular cytokine staining (DC stimulators), post-culture CSFE assay (proliferation), Chromium release cytotoxicity assays, MLR	
Li *et al.*, 2004 63	Identification of new Th 15-mer peptides by ELISPOT screening of 15-mer peptide panels for the generation of a library	Peptide library could be used to generate CMV-specific CD4 T cell lines	Proliferation of library-generated T cells in response to peptides, CMV lysate, protein derivative, and allo cells	No further assays to test T cell function

CMV-specific T cells can be selected directly from fresh PBMC, and utilised without further modifications, or following non-specific expansion to increase the available cell numbers [58] (Figure 1.2). Alternatively, antigen-specific T cells can be enriched through antigen-driven cultures starting with unselected T cells. T cells generated in this way can be used directly, or following an antigen-specific selection process (Figure 1.2).

A relatively clearly defined cell product can be achieved by selecting T cells specific for single peptides of CMV [49;50]. For this, prior knowledge of the immunodominant peptides is required. This is helped by the fact that the CD8-mediated cellular immune response against CMV can be very skewed towards few immunodominant peptide-MHC complexes [64]. For the most common human HLA types at least, several immunodominant peptides have been identified [65-71]. HLA-tetramer technology lends itself to the isolation of peptide-specific CD8 T cells before or after antigen-driven culture, such as proposed by Szmania *et al.*[49]. While this approach avoids the culturing of virus, there is a theoretical risk of posing a selective pressure on CMV to evade an immune response directed against a single or low numbers of target epitopes. The approach is also limited to few known peptides, and restricted to donors with the HLA-types of the tetramers used. Whilst HLApeptide-tetramers bind CD8 T cells specific for single peptide-HLA combinations, pools of overlapping peptides, spanning a whole protein can be manufactured and utilised to stimulate a broader T cell response in culture. In this case, no prior knowledge of immunodominant epitopes for specific HLA types is required, and the patient's tissue type does not represent a limitation. Whole recombinant proteins can be used for the same purpose, but require processing by APC and in most settings would generate predominantly CMV-specific CD4 T cells. A hybrid recombinant protein of pp65 and IE-1, the two most immunodominant proteins of CMV has been tested *in vitro* to generate CMV-specific CD4 and CD8 T cells [57].

It became clear early on that CMV-specific CD4 T cells play an important role in the maintenance of CMV-CTL, and may be necessary for the development of immunological memory. They can also mediate cytotoxicity towards CMV infected cells in their own right [60;72]. Short peptides efficiently stimulate CD8+ CTL, whereas proteins are more effective at stimulating CD4 T cells.

The isolation of T cells producing cytokines in response to *in vitro* exposure to any chosen antigen type, i.e. peptide, protein, or lysate [58], can capture CD8 and CD4 T cells, either from fresh PBMC, or following antigen-driven T cell culture. For this purpose cytokines are co-localised to cell surface molecules by an antibody matrix and cytokine-secreting cells then identified by cytokine-specific mabs (Figure 1.3).

The advantages and limitations of antigen-specific T cell populations isolated by either HLA-tetramers, or by cytokine secretion capture are addressed in greater detail in chapter 4.

In addition to infection with CMV and antigen-pulsing, genetic modification of APC to express virus antigens to T cells is possible (reviewed by Andre-Schmutz et al. Hum. Immunol. 2004 [73]).

An EBV vector with added CMV genes can immortalise B cells. These can then induce bi-specific CTL [53;74]. Poxiviruses can be utilised to transiently transfer genes into PBMC, long enough for antigen presentation to autologous T cells [18]. DC can also efficiently be transduced by adeno- [75] or lentiviruses [76-78]. Adenovirus is itself immunogenic, which might benefit SCT recipients at risk of both adenovirus and CMV infections.

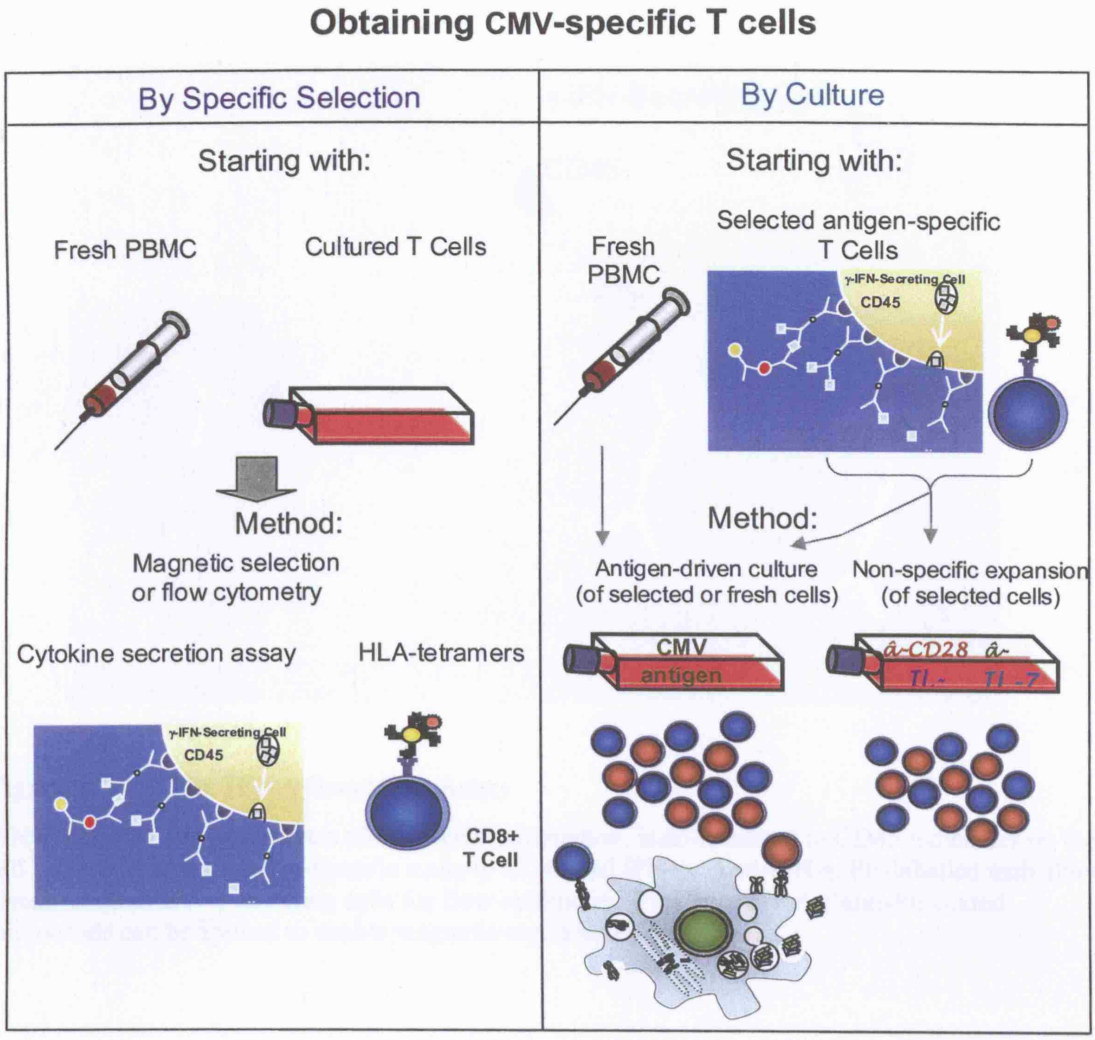

Figure 1.2 Diagrammatic Representation of Different Approaches to the Generation and/or Isolation of CMV-Specific T Cells from CMV Seropositive Donor Blood.

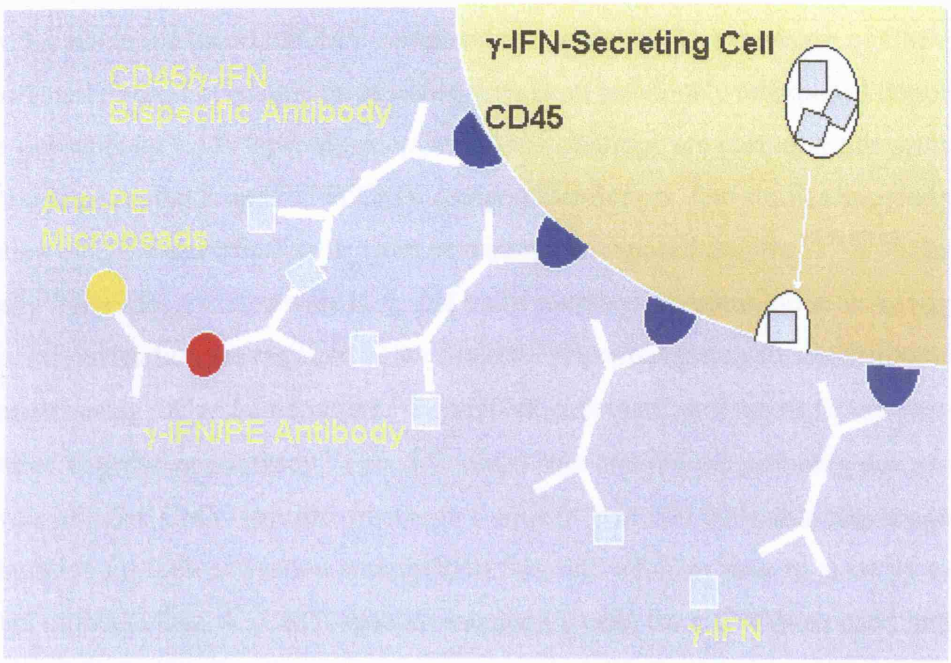

Figure 1.3 The IFN-γ Secretion Assay

IFN-γ, secreted by lymphocytes after specific stimulation, is co-localised to CD45 molecules on the cell surface by a matrix of bi-specific mabs to CD45 and IFN-γ. Anti-IFN-γ, PE-labelled mab allow visualisation of IFN-γ-secreting cells for flow cytometry. Finally, a layer of anti-PE coated microbeads can be applied to enable magnetic-activated cell sorting.

1.8.3 Generation of CMV-Specific T Cell Lines from CMV Seronegative Donors

The majority of studies make use of the relatively high levels of readily activated CMV-specific T cells in the blood of CMV seropositive donors for the expansion of CMV-specific T cell lines. For recipients of transplants from previously unexposed donors, cellular products for CMV-specific adoptive immunotherapy are currently not available. Using the same methods applied to CMV seropositive donors, few studies succeeded in the generation of CMV-specific T cells from previously unexposed donors [49;50;52;60]. In all but one study [60] the success rates were low, and more rounds of re-stimulation were required, significantly extending the required culture period. Tazumes group succeeded in 4/6 experiments using DC matured with LPS and TNF-α as APC, but more experiments would be required to prove consistency. The difficulties encountered are probably due to the very low levels of naïve CMV-specific precursor T cells (< 1 in 100 000), that require greater co-stimulation for their activation and proliferation, and a longer time to grow up to sufficient numbers than the CMV-specific memory T cells from CMV-exposed individuals. The activation of naïve T cells may require more specialised culture conditions ensuring stronger co-stimulation.

Induction of primary T cell responses has been successful using immunologically irrelevant antigens, such as KLH [79;80]. Our attempts to expand CMV-specific T cells from CMV seronegative donors in culture are described in chapter 5.

Novel approaches to address this problem include the generation of T cells that have been genetically modified to express virus-specific T cell receptors [81-84]. However, problems regarding their rapid loss of function still need to be overcome.

Another way around this problem is to vaccinate the donor against CMV, for example using recombinant canarypox-pp65 [85] to be able to then generate CMV-specific T cells from donor blood *in vitro*.

1.9 Aims of this Work

Our T cell lines generated during a 2-3 week co-culture with autologous CMV lysate-pulsed MoDC [56] can be expected to cover the whole range of CMV epitope specificities and contain both CD8+ and CD4+ T cells (figure 1.4). CMV antigen-specific T cell

proliferation and HLA-restricted lysis of CMV antigen-pulsed or CMV-infected target cells by post-culture T cells have previously been demonstrated *in vitro* [56] (figure 1.5). This cell product has already shown some effectiveness in an ongoing immunotherapy protocol. Low doses of cells appear to be effective at restoring an immune response against CMV if used in pre-emptive adoptive transfers [46]. On the other hand, the culture output is still poorly defined and variable.

This dissertation deals with further characterisation of the cell products achieved with this culture method from CMV seropositive donors, and experiments leading to greater optimisation of the culture conditions will be described. The characterisation of cultured CMV-specific T cells also provides the opportunity to learn more about the nature of CMV-specific T cell responses in healthy infected persons.

In addition to this, the optimised methods are applied to cultures from CMV seronegative donors to generate *in vitro* primary CMV-specific T cell responses.

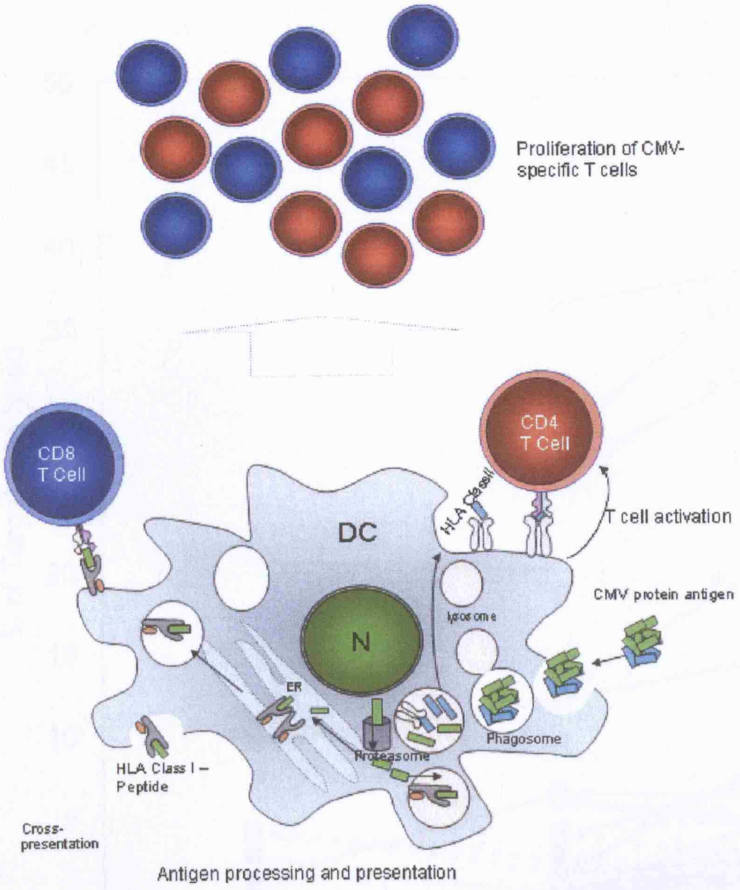

Figure 1.4 Generation of CMV-Specific T Cells in Culture Using Autologous Antigen-Pulsed DC

Autologous DC pulsed with CMV antigen present antigen to CD4- and CD8- expressing T lymphocytes. DC endocytose and process protein antigens, and present Peptides on class II molecules to CD4 T cells. DC can also cross-present exogenous antigen on HLA class I molecules to CD8 lymphocytes. Alternatively free peptides in solution can directly bind to HLA class I and class II molecules on the DC plasma membrane. T cells proliferate in response to activation through the antigen-presenting DC.

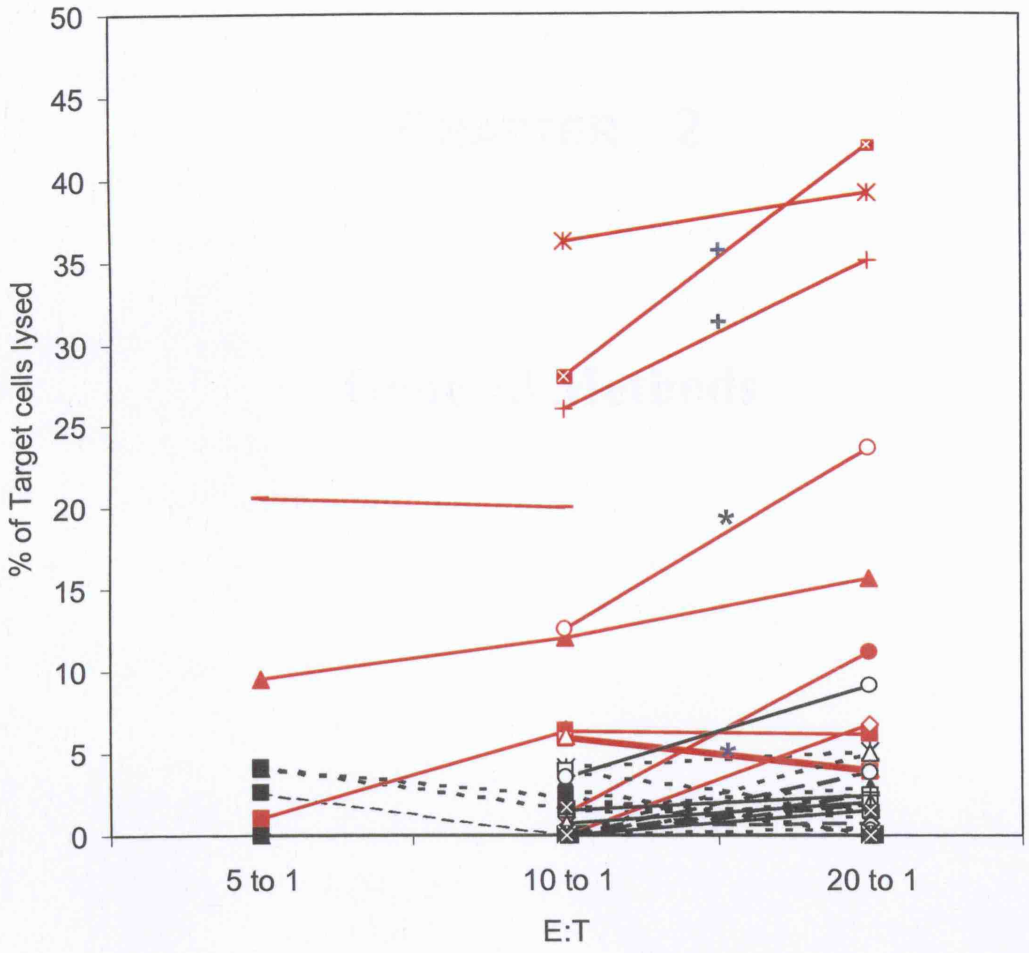

Figure 1.5 Lysis of Target Cells by the Output from CMV Antigen-Driven T Cell Cultures: LDH Release Cytotoxicity Assays.

The graph shows pooled results of separate experiments with cells from 12 different donors (symbols). Post-CMV antigen-driven effector cells (E) were co-cultured with autologous or allogeneic target cells (T) at various ratios (X-axis). After 4h of co-culture, the relative amount of LDH release from lysed target cells was determined through the addition of a chromogenic substrate for LDH.
Effector cells: CMV antigen-driven culture output from 2-3-week cultures. **Red lines**, experimental target cells: Autologous fibroblasts or DC infected with CMV or pulsed with CMV antigen. **Black lines**, control target cells: uninfected/unpulsed autologous target cells (__ __ __) , uninfected/unpulsed allogeneic target cells (_____), infected/antigen-pulsed allogeneic target cells (- - - -). **Blue symbols** above experimental graphs indicate different target cell types used: +, DC infected with CMV; *, DC pulsed with CMV antigen. Where there is no blue symbol, fibroblasts were infected with CMV.

CHAPTER 2

General Methods

2.1 Materials

See appendix (i) for a list of chemicals and consumables and their manufacturers.

2.2 Blood Donors

Blood was collected from 35 healthy human adult volunteers, whose age ranged from 23 to 50. CMV serostatus was determined by complement fixation test, qPCR, or both. Eleven volunteers were CMV negative, the remaining 24 volunteers were CMV positive.

2.3 Obtaining Human Serum from Peripheral Blood

Tubes of peripheral blood without additives were allowed to clot, and centrifuged at 700g for 15min. The serum layer was moved to a fresh tube leaving the clot behind, followed by another round of centrifugation at the same force for 10min to sediment any remaining debris. The serum was then filtered though a 20μm pore-size syringe filter and heat inactivated at 58°C for 1h in a waterbath to inactivate complement. Human serum was stored at –20°C.

2.4 Isolation of PBMC by Differential Centrifugation

PBMC were separated from red blood cells and neutrophils by gradient centrifugation through Ficoll. Heparinised peripheral blood was diluted 1:1 with PBS w/o Ca+ and Mg+ (PBS w/o). Each 30ml of diluted blood was layered onto 20ml Ficoll in 50ml Falcon tubes and centrifuged at 600g for 25min with minimum brake settings. The interphase layer containing the PBMC was transferred to a fresh 50ml tube. To remove contaminating Ficoll from PBMC, the tube was filled with PBS w/o Ca/Mg, centrifuged at 600g for 10min, and the supernatant discarded. The cell pellet was re-suspended in 50ml PBS w/o. Platelets were removed by gentle centrifugation at 260g for 10min, resulting in the sedimentation of PBMC with the platelets remaining in suspension. The supernatant containing the platelets was discarded, and PBMC were re-suspended in minimum volume.

2.5 Cryopreservation and Thawing of Cells

2.5.1 Cryopreservation of Cell Suspensions

PBMC, lymphocytes, and MoDC were all cryopreserved by the same method. Freezing solution was first prepared at double the final concentration required: X Vivo 20 medium, 40% autologous HS or FCS, 20% DMSO. In order to prevent damage to serum components caused by the exothermic reaction with DMSO, the serum was pre-cooled on ice. DMSO was added first to cold serum before addition of the medium. For cryopreservation, cells suspended in X Vivo 20 medium were diluted 50:50 with the freezing solution to give the final concentration of 20% serum, 10% DMSO, and frozen in 1ml aliquots in cryovials. Wrapping the vials in polystyrene, or using an isopropanol-filled freezing container ensured a gradual decrease in temperature by approximately 1°C/min down to -80°C. For long-term storage cryovials were moved from –80°C into liquid nitrogen.

2.5.2 Cell Thawing

Cells were thawed rapidly in a 37°C waterbath. The cells were gently rehydrated by the gradual addition of X vivo 20 at 10 times the volume of the freezing medium. The diluted cell suspension was centrifuged at 260g for 5min. The supernatant containing the freezing solution was discarded and the cells resuspended in fresh tissue culture medium.

2.6 Generation of Monocyte-Derived DC

2.6.1 Isolation of Monocytes

Monocytes were isolated from the PBMC by either one of the following methods:

2.6.1.1 Magnetic Activated Cell Sorting (MACS) of CD14+ Monocytes

This method was chosen when a pure population of Mo was required. Labelling and magnetic enrichment of CD14+ cells was carried out according to the protocol provided by the manufacturer. PBMC were resuspended in 80µl cold MACS buffer (PBS, 2mM EDTA, 0.5% human serum albumin) per 10 million cells. 20µl of anti-CD14 microbeads per 10

million PBMC were added. The cell suspension was incubated at 6-12°C for 15min. After the incubation, 10-20 times the labelling volume of cold MACS buffer was added, followed by centrifugation at 300g for 10min at 4-8°C. After discarding the supernatant, the cell pellet was re-suspended in 3ml of MACS buffer before enrichment by MACS.
For magnetic selection an LS/VS MACS column was placed into a VarioMACS magnet and prepared by applying 3ml of MACS buffer. After the buffer had passed through the column, the cell suspension was applied, followed by three washes with 3ml of cold MACS buffer. The CD14 depleted fraction was collected into a 15ml tube, centrifuged at 300g for 5 min and cryopreserved (section 2.5). After completion of the final washing step, the column containing the CD14+ fraction was removed from the magnet, 3ml of cold MACS buffer was applied to the column, and the plunger was used to flush out the cells. CD14+ cells were collected into a 15 ml tube and centrifuged at 300g for 5min. Alternatively, CD14+ cells were flushed out of the column with 3ml of culture media instead of buffer, thus avoiding centrifugation before culture.

2.6.1.2 Enrichment of Monocytes from PBMC by Adherence

PBMC were re-suspended in X Vivo 20 medium and seeded into tissue culture flasks or plates at a maximum of $4x10^5/cm^2$. Monocytes were allowed to adhere to plastic for a minimum 1.5h in a humidified incubator with 5% CO_2 at 37°C. After the incubation, the non-adherent fraction was collected into a 50ml tube. Adherent cells were washed by vigorous pipetting with PBS w/o Ca/Mgto remove as many non-adherent cells as possible. The washing step was repeated four times. X vivo 20 containing 10% autologous HS, 100ng/ml GM-CSF, 100ng/ml IL-4 was added to the adherent monocytes. The non-adherent fraction containing the lymphocytes was centrifuged at 300g for 5min. The pellet was re-suspended in freezing solution and cryopreserved (section 2.5).

2.6.2 Differentiation of Monocytes into Immature MoDC *in vitro*

Monocytes were cultured at a density of about $1x10^5/cm^2$ in X Vivo 20, 10% autologous HS, 100ng/ml GMCSF, 100ng/ml IL-4 in a humidified incubator with 5% CO_2 at 37°C for 7 days. During the differentiation the monocytes become less adherent, reduce slightly in number, undergo an about 4-fold size-increase, and start to display typical DC morphology.

2.6.3 DC Loading with Antigen

On day 4 of MoDC differentiation DC were loaded with CMV or control antigen. CMV or control lysate was reconstituted in 1ml X Vivo 20 medium and applied to the MoDC cultures in a 1: 20 dilution.

2.6.4 DC Maturation

MoDC cultured as inducers for antigen-specific T cell proliferation were matured with CD40L where indicated. 300ng/ml CD40L was added to differentiating MoDC on day 4 of differentiation, at least 3h after CMV antigen loading (section 2.6.3).

2.6.5 Cytocentrifuge Preparations

Cytocentrifuge preparations were made by suspending $2x10^4$ DC in 200 µl of heat inactivated FCS in a cytocentrifuge cup. The cells were spun onto slides at 300 rpm for 10 minutes and air dried. Slides were fixed in methanol and stained with the M-G-G stain.

2.7 *In vitro* Expansion of CMV-Specific T Lymphocytes for Adoptive Transfer

2.7.1 Co-culture of CMV Antigen-Pulsed MoDC with Autologous PBL

50-100ml heparinised peripheral blood was the starting material. PBMC were obtained as described in section 2.4. Monocytes for the generation of MoDC were obtained by adherence selection (section 2.6.1.2) in two filter-top tissue culture flasks ($25cm^2$ and $75cm^2$), into which PBMC were divided proportionally. MoDC in both flasks were loaded with CMV antigen (section 2.6.3). MoDC in the smaller flask were matured with CD40L (section 2.6.4). At the end of the 7-day differentiation the MoDC in the larger flasks were cryopreserved for later use in re-stimulation. Co-cultures with autologous lymphocytes were set up in the smaller flask. The GM-CSF and IL-4-containing medium was replaced with fresh X Vivo 20 medium, 10% autologous HS. Autologous lymphocytes (the non-adherent fraction, section 2.6.1.2) were thawed (section 2.5.2) and added to the CMV antigen-pulsed, matured MoDC at a maximum density of $2x10^6$/ml. Co-cultures were

maintained for 14 days. Half the medium was exchanged when required, as indicated by a media colour change. Proliferation of CMV-specific T cells is usually detectable by microscopy from about 3 days into co-culture. T cells begin to cluster around DC, before increasing in number. From day 4 to about day 9, T cells tend to grow rapidly, then proliferation slows down and finally the cell number decreases. Cultures were re-stimulated at weekly intervals by addition of thawed antigen-pulsed MoDC to the culture. From day 10 onwards, 20 U/ml IL-2 were added every second day. Addition of IL-2 at this late time point is thought to specifically maintain the activated, CMV-specific T cells.

2.7.2 Harvest and Cryopreservation of *in vitro* Expanded CMV-Specific T Cells

After 14 days of co-culture with MoDC, the lymphocytes were harvested and layered onto Ficoll. Any dead cell debris was separated from life cells by differential centrifugation (section 2.4). The interphase layer, containing mostly alive cells was moved into a fresh tube. Contaminating Ficoll was discarded after a wash with PBS w/o Ca, Mg followed by sedimentation of the cells at 600g for 10min. The cells were re-suspended in X vivo 20 medium, counted, and cryopreserved (section 2.5.1).

2.8 ^{3}H-Thymidine Incorporation Assay

Incorporation of tritiated Thymidine into the nucleic acids of growing cells is an established way of measuring cell proliferation. The assay conditions were adapted here to detect the maximum possible difference in T cell proliferation induced by CMV antigen-pulsed MoDC and control antigen- or unpulsed MoDC. The assay was optimised for detection of differences within the same assay, but direct quantitative inter-assay comparisons of unnormalised data were not permissible.

Mo selected by CD14-MACS, or by adherence, were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 (see section 2.6.2) for 7 days, and pulsed with CMV antigen, control antigen, or no antigen. After the 7 days, MoDC were collected, sedimented at 300g for 5min, and the supernatant containing cytokines and antigen was discarded. The resulting pellet of MoDC was re-suspended in X vivo-20, 10% autologous serum. MoDC have no proliferative potential, unlike contaminating cell types also present in the MoDC cultures [86]. Therefore irradiation of MoDC (25Gy) derived from adherence-selected Mo,

which generally contained high numbers of contaminating cells, was required to eliminate background proliferation. CD14-selection gave rise to a purer population of MoDC, and irradiation was not as crucial. Figure 2.1 shows no extra incorporation of ^{3}H-Thymidine in cultures containing un-irradiated DC derived from CD-14-selected monocytes. Triplicate co-cultures of typically 10^4 MoDC and 1-2x10^5 autologous PBL/well were set up in round-bottom 96-well plates. The cultures were incubated in a humidified chamber at 37°C with 5% CO_2. The plates were checked in an inverted microscope for signs of proliferation on a daily basis to determine the best time for termination of the assay. T cell proliferation was clearly visible in CMV antigen wells, as shown in figure 2.2, but before T cells outgrew the media, 1μCi ^{3}H-Thymidine was added to each well for the final 16h before harvest. Cultures from CMV seropositive donors were typically harvested on day 6. During harvest, solid components, including nucleic acids, from each well were harvested onto a filtermat using an automated harvester. A scintillant sheet was melted onto the filtermat at 100°C. The scintillant was allowed to cool and solidify. Then tritium was detected by a scintillation counter taking 10-second readings for each well.

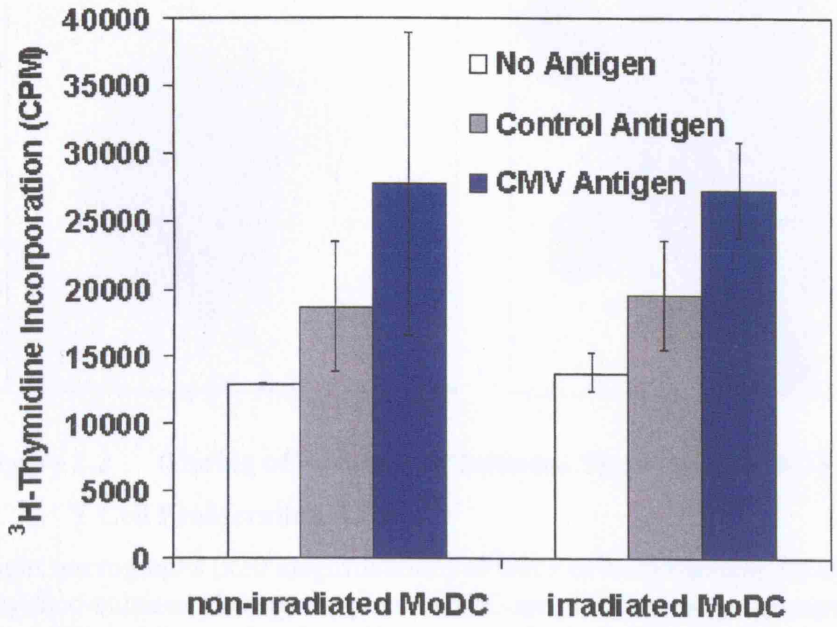

Figure 2.1 DC Derived from Monocytes Isolated by MACS: Irradiation of DC does not Affect Levels of ^{3}H-Thymidine Incorporation.

Monocytes were positively selected from PBMC from one donor (CMV seronegative) by MACS with CD-14 microbeads. Monocytes were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 for 7 days. Irradiated or non-irradiated MoDC, pulsed with CMV antigen (blue bars), control antigen (grey bars), or left unpulsed (white bars) were co-cultured with autologous T cells for 9 days in triplicate cultures. Incorporation of ^{3}H-Thymidine during the last 16h was measured by scintillation counting.

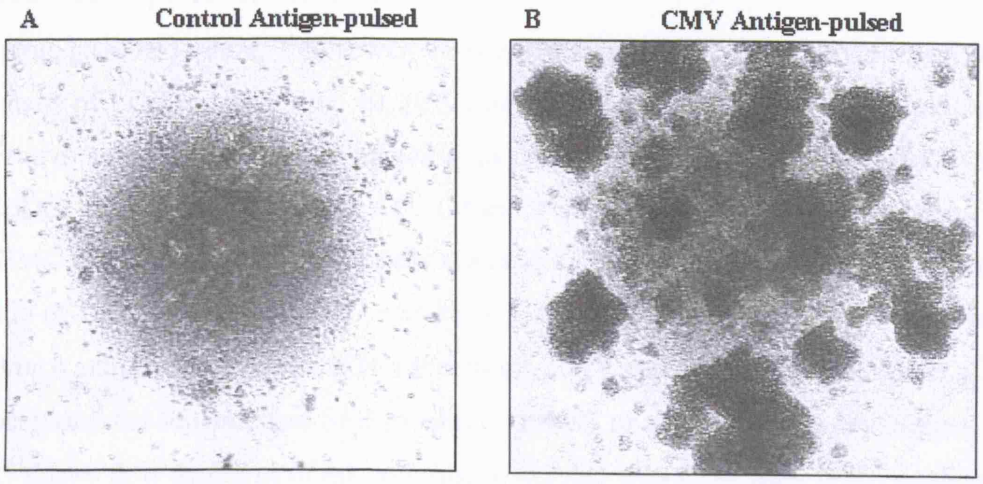

Figure 2.2 Timing of Addition of Tritiated Thymidine to DC-PBL Co-Cultures for T Cell Proliferation Assays

Light micrographs (x20 magnification) of wells of round-bottom 96-well plate containing day-6 co-cultures of antigen-pulsed MoDC and autologous lymphocytes on the day of ^{3}H-Thymidine addition. **(A)** Control antigen-pulsed MoDC. **(B)** CMV antigen-pulsed MoDC, showing clusters of proliferating T cells. ^{3}H-Thymidine was added when CMV-specific T cell proliferation was clearly visible, as shown here, and before colour change in culture media.

2.9 Immunophenotyping of Pre-Culture and Cultured Lymphocytes

PBMC or post-culture lymphocytes were re-suspended in X vivo 20 medium. Direct staining of 5-10x10^{5} cells with fluorochrome-conjugated monoclonal antibodies was carried out in 96-well plates in 100µl volumes. 10µl of triCHROME (anti-CD3-PC5, anti-CD4-RD1, anti-CD8-FITC) for simultaneous staining of three T cell markers were added to the cells on ice, and left to incubate for 30min on ice in the dark. To wash off unbound antibody, after the incubation, the wells were filled with cold PBS w/o Ca+ Mg+, 2% FCS. The plates were centrifuged for 5min at 260g and the supernatant discarded. The resulting pellets were resuspended in PBS, 2%FCS, 1% Formaldehyde and stored at 6-12°C until analysis by flow Cytometry (see section 2.13).

2.10 IFN-γ Secretion Assay

Before gamma-capture, T cells were re-stimulated overnight with CMV antigen at a cell density of $1x10^7$/ml in X Vivo 20, 10% autologous HS. T cells from CMV antigen-driven cultures were restimulated with autologous CMV- or control antigen-pulsed PBMC or MoDC, where indicated. Since APC (Monocytes) only constitute about one third of PBMC, MoDC were added to T cells at a ratio of 1:20 whereas PBMC were added at a ratio of 1:5. Uncultured PBMC, serving as controls, were re-stimulated directly with control antigen (background IFN-γ secretion), CMV antigen (pre-culture levels of IFN-γ secretion), the superantigen SEB (positive control), or not exposed to any antigen. Figure 2.3 shows flow diagrams of the controls, co-stained with CD4, after IFN-γ capture.
For Gamma-capture the manufacturer's protocol was followed. After the 16-h restimulation pre-culture PBMC or post-culture lymphocytes were collected into 50ml tubes. 10ml of cold MACS buffer (PBS, 0.5% HS, 20mM EDTA) were added, and the cells were sedimented at 300g for 10min at 6°C. After centrifugation the supernatant was completely removed and the cells were resuspended in 80µl of cold MACS buffer. 20µl of IFN-γ catch reagent was then added, followed by a 5-minute incubation on ice. 30ml of warm (37°C) X vivo 20 medium, 10% FCS were added. The cells were incubated at 37°C with light shaking for 45min. After the incubation, the cells were washed by filling the tubes with cold Miltenyi buffer, and centrifuged at 300g at 6°C for 10min. The supernatant was removed completely. The washing step was repeated once. After complete removal of the supernatant, the cells were re-suspended in 80µl MACS buffer. 20µl IFN-γ detection reagent (conjugated to PE), and 10µl FITC- or APC-conjugated monoclonal antibodies against the T cell markers CD4/8 were added. The cells were incubated for staining on ice for 10min. After the incubation any unbound antibody was washed off by adding 10ml of cold MACS buffer followed centrifugation at 300g at 6°C for 10min. The supernatant was removed completely, and the labelled cells were either resuspended in 500µl of cold MACS buffer, transferred to FACS tubes and kept on ice until analysis/sorting by flow cytometry. Alternatively, some of the fluorescently stained cells were labelled with immunomagnetic beads for subsequent separation by MACS.

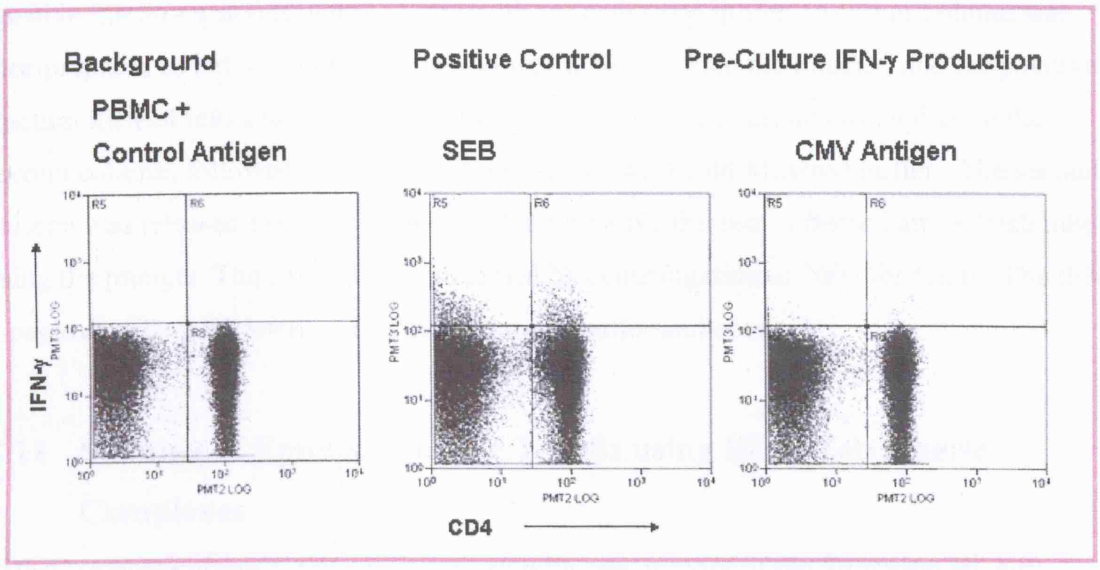

Figure 2.3 Controls for IFN-γ Secretion Assay.

Determination of background frequencies of IFN-γ secreting T cells: Autologous PBMC were pulsed with control antigen for 16h. Positive control: PBMC were pulsed with SEB for 16h. Pre-culture IFN-γ secreting T cells: PBMC were pulsed with CMV antigen for 16h. Background frequencies of IFN-γ secreting T cells were subtracted from other controls and all experimental values.

2.10.1 Immunomagnetic labelling of IFN-γ-secreting T cells

The pellet of IFN-γ-PE-labelled cells was re-suspended in 80µl cold MACS buffer. 20µl anti-PE microbeads were added, followed by an incubation of 10min at 6-8°C. After the incubation, 10ml of cold MACS buffer was added. Excess beads were washed off by a 10min centrifugation at 300g at 6°C. The supernatant was discarded, and the pellet was re-suspended in 500µl cold MACS buffer. At this point the cells were ready to be applied to an MS column for MACS.

2.10.2 Enrichment of IFN-γ Secreting T Lymphocytes by MACS

Were indicated, IFN-γ-secreting cells labelled with immunomagnetic beads were sequentially enriched through two MS columns. The first column was placed in a miniMACS magnet and prepared by applying 0.5ml of cold MACS buffer. After allowing the buffer to flow through the column, the suspension of IFN-γ-labelled cells was applied and the IFN-γ negative fraction collected into a tube. The column containing the IFN-γ

positive fraction was rinsed three times with cold Miltenyi buffer. Another column was then prepared as before. The first column was removed from the magnet, and the positive fraction flushed into a tube using the plunger. The positive fraction was applied to the second column, followed by a further three washes with cold Miltenyi buffer. The second column was released from the magnet, and the positive fraction collected into a fresh tube using the plunger. The cells were sedimented by centrifugation at 260g for 5min. The IFN-γ-positive cells were resuspended in medium or buffer and counted.

2.11 Staining of Epitope-Specific T Cells using HLA-Tetrameric Complexes

PBMC or post-culture T cells were stained with one or more of the following HLA-tetrameric complexes according the donor's HLA type: HLA A*A0201-tetramer-CMVpp65$_{NLVPMVATV}$, HLA A*0201-tetramer-IE1$_{VLEETSVML}$, HLA-B*0702-tetramer-pp65$_{TPRVTGGGAM}$, HLA B*0702-tetramer-CMVpp65$_{RPHERNGFTVL}$, HLA A*2402-tetramer-CMVpp65$_{QYDPVAALF}$, and HLA A*2402-CMVpp65$_{VYALPLKML}$. All tetramers were PE-conjugated to for detection. The cells were thawed as described in section 2.5.2. After the wash the cells were re-suspended in minimum volume in 15ml tubes to remove the freezing medium. 2μl of HLA-tetramers were added per 1x10^6 cells, followed by a 30-min incubation in a 37°C waterbath. After the incubation, the cells were placed on ice, 10μl anti-CD8-FITC mabwere added per 1x10^6 cells. The cells were incubated for 20min on ice. Unbound antibody and tetramers were washed off by adding 10ml PBS, 0.5% bovine serum albumin, 0.01% sodium azide, followed by centrifugation at 300g for 5min. The supernatant was removed, and the cell pellet re-suspended in 500μl PBS, 0.5mg/ml bovine serum albumin, 0.001% sodium azide and transferred to a FACS tube for immediate flow cytometry.

2.12 Non-Specific Expansion of Magnetically Selected Post-Culture IFN-γ+ T Cells

Post-culture cells were enriched for IFN-γ-secreting cells by MACS (section 2.10.2). IFN-γ+ cells were non-specifically expanded according to the method described by Bissinger et al. [58] with modifications. IFN-γ+ cells were resuspended in X Vivo 20 medium, 10% FCS, 50U/ml IL-2 and seeded at $2x10^4$/well in 4 wells of a 24-well plate together with $1.2x10^6$ irradiated (25Gy) allogeneic PBMC/well as feeder cells. Additionally, two of the wells contained 10ng/ml IL-7. Half the medium was replaced with fresh, cytokine-containing medium every other day. On day 7, cells were counted and reseeded at $1.2x10^5$ T cells and $1.2x10^6$ feeder cells/well. Excess cells were cryopreserved for analysis at a later time.

2.13 Immunophenotyping of MoDC

MoDC were collected from tissue culture flasks into 50ml tubes by vigorous pipetting, and several rinses with PBS w/o Ca+, Mg+. The cells were sedimented by centrifugation at 260g for 5min. The supernatant was discarded and the MoDC were re-suspended at 0.5-$1x10^6$/ml in PBS w/o Ca+, Mg+, 50% AB serum. MoDC were stained with monoclonal antibodies against surface markers CD14, CD86, CD40, CD83, HLA-DP, -DQ, -DR, or with isotype control mabs in 100µl volumes in 96-well plates for 30min. DC were either double or single stained with mab conjugated to FITC or PE. After the incubation, unbound antibody was removed by filling the wells PBS, 2% FCS followed by centrifugation at 260g for 5min. The supernatant was discarded, and the cells re-suspended in 500µl PBS, 2% FCS, 1% formaldehyde, and transferred to FACS tubes for later analysis by flow cytometry.

2.14 Flow Cytometry – Analysis and Cell Sorting

Cells were analysed using an EPICS Elite flow cytometer (BeckmanCoulter). Cells were first gated by size and granularity to narrow down the population of interest, using forward and side scatter, in order to exclude other cells and debris from the analysis. Cell doublets were excluded from the analysis on a histogram of forward scatter mean versus forward scatter. A minimum of 10 000 total events were collected for CD3/4/8 phenotyping of T cell populations, and CD4 and CD8 T cell frequencies were expressed as percentages of CD3+ cells.

A minimum 100 000 events were collected for lymphocytes stained for IFN-γ secretion or with HLA-CMVpeptide-tetramers.

For DC phenotyping a minimum of 5000 events were collected.

For the analysis of lymphocytes stained for IFN-γ and surface markers as described in section 2.10, approximately 20ng/ml PI was added to each tube just prior to analysis, to allow the exclusion of dead and dying cells, which non-specifically stain with anti-PE beads. The PI excluding fraction constituted the population of live cells.

Background levels of IFN-γ-secreting T cells were determined by stimulating PBMC with control antigen. The background was subtracted from all experimental values. The frequency of IFN-γ-secreting cells was expressed as a percentage of CD4+ or CD8+ T cells. Cells were sorted according to expression of IFN-γ and by their CD4 or CD8 phenotype.

For DC phenotyping, cells stained with the isotype control mabs served as negative controls. Frequencies of cells expressing surface markers above the negative control were expressed as a percentage of total MoDC. The mean fluorescence intensity was an indicator of levels of surface marker expression/ cell. The arbitrary units of this measurement varied widely between experiments, according to the strength of laser used.

The frequency of HLA-tetramer positive cells was expressed as percentage of CD8+ T cells. CD8+/HLA-tetramer positive cells were sorted.

2.15 Statistical Analysis

For very small sample sizes ($n<6$) descriptive statistics such as mean and standard deviation, or median and range were provided without further analysis.

Means of small samples ($size:5<n<20$) from populations with normal or near normal distributions were compared by the student's t-test (2n-2 df, or paired student's t-test for paired data (n-1 df), using MedCalc software.

Apart from the usual variability in labelling efficiency, the use of two different flow cytometers with different settings and laser powers further complicated compatibility of MFI data across different experiments. However, paired data was generated for each experiment (treated DC versus untreated DC). To make these data comparable across experiments the MFI values for untreated DC were normalised to 1. The differences between untreated and treated DC were then analysed by the t-test (N.H.: mean = not different from 1, n-1 df) using Excel software.

Generally, for all t-tests, p values $<0.05\%$ were considered significant.

^{3}H-Thymidine incorporation proliferation assays generated triplicate data points for each condition. These data were usually expressed in bar charts showing the average of each triplet with their SDs shown as error bars (e.g. Figure 2.1). In specific cases, where one value of the triplicate was very different from the other two, the data was expressed as median and range, to give a more accurate representation of the result (e.g. Figure 5.2A).

To be confident that CMV-induced responses were significantly different from background responses (control antigen- or no antigen-induced responses), they had to be separated by a minimum of 2.5SD, i.e.:

(mean 'control response' + 2.5SD) < (mean 'experimental response' – SD).

When comparing CMV antigen-induced (positive) responses to one another, they were considered to differ significantly if there was no overlap between SDs:

(mean 'lesser response' +SD) < (mean 'greater response' –SD).

2.16 TCR CDR3-Spectratyping

RNA was extracted from PBMC, *in vitro* expanded CMV-specific T cells, IFN-γ+, or Tet+ T cells. Complementary (c)DNA was generated and amplified by the polymerase chain reaction (PCR) essentially according to the method published by Maslanka *et al.*[87].

2.16.1 RNA Extraction

A minimum of 10 000 cells were lysed by the addition of 1ml of Ultraspec RNA emulsion. The lysates were stored in 1.5ml tubes at -80°C until further processing. Lysates were thawed on ice, 200µl of chloroform were added and thoroughly mixed with the lysate. Centrifugation at 12000g for 15min at 4°C resulted in phase-separation. The aqueous phase containing the RNA was transferred to a fresh 1.5ml tube. For RNA precipitation, 500µl isopropanol were added. Glycogen was added to a final concentration of 5µg/ml, for co-precipitation with the RNA to enable later visualisation of the RNA pellet. The precipitated RNA/glycogen was centrifuged at 12000g for 10min at 4°C. The supernatant was discarded, and the pellet washed by adding 1ml of 70% ethanol, followed by centrifugation at 7500g, 4°C for 5min. The supernatant was removed completely. The RNA pellet was typically resuspended in 30µl sterile water for injection. The concentration of RNA was calculated from spectrophotometer readings at 260nm wavelength using the molar extinction coefficient of RNA. In the case of low numbers, e.g. sorted HLA-tetramer+ or γ-IFN+ cells, the RNA pellet was resuspended in 10µl sterile water, and reverse transcribed in a single reaction, without measuring RNA concentration.

2.16.2 Synthesis of cDNA

1µg of RNA, or RNA derived from a minimum of 10 000 PBMC was reverse transcribed to cDNA in a 30µl reaction volume, containing first strand buffer (Invitrogen), 10mM DTT, 0.5mM each deoxyribonucleotide triphosphate (dNTP) (dATP, dCTP, dGTP, dTTP), 1x hexanucleotide mix, 60U/µl Superscript reverse transcriptase. In the case of low cell number (e.g.: sorted tetramer+ or IFN-γ+ cells) the total amount of RNA (9µl) was reverse transcribed in a single reaction.

2.16.3 Polymerase Chain Reaction for TCR CDR3 Spectratyping

A panel of 24 variable region-beta (BV) gene subfamily-specific primers [87] were combined with a single fluorescently labelled constant-beta (BC) primer [88] (3`-conjugated to FAM or wellred4 dyes) to amplify across the functionally rearranged constant-variable junctions of the TCR CDR3. Some of the BV primers were paired in multiplex reactions, giving rise to one shorter and one longer PCR product (table 2.1). The PCR-mix contained Geneamp PCR buffer, 2mM magnesium chloride, 0.2mM of each dNTP, 0.5μM of each primer, 1-2μl cDNA (equivalent to 770-25000 cells). For the hotstart 0.5U Amplitaq DNA polymerase were added after a 5-minute denaturation at 95°C, bringing the final volume to 20μl. TCR CDR3 DNA was amplified by 33 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds. This was followed by a final extension at 72°C for 5 minutes.

Shorter PCR product	Longer PCR product
BV 5.1	BV 1
BV 2	BV 12
BV 8	BV 3
BV 4	BV 5.3
BV 13	BV 7
BV 9	BV 14
BV 11	BV 20
BV 17	BV 15
BV 16	BV 21
BV 22	BV 24
BV 18	BV 23
BV 6.1	-
BV 6.2	-

Table 2.1 Combination of BV Primers in Duplex PCR Reactions.

2.16.4 Fragment Analysis of PCR-Products

PCR products were electrophoresed and analysed either on the ABI 310 automated sequencer (Applied Biosystems), or the CEQ 8000 genetic analysis system.

2.16.4.1 Genscan Analysis on ABI 310

1µL of the PCR product and 1µl Tamra-conjugated 500 bp size standard were denatured at 95°C for 5min in 12µL formamide in 0.5ml tubes, and electrophoresed through performance optimized polymer 4 on the ABI 110 automated sequencer. Genescan 2.1 software was used to analyze the data.

2.16.4.2 Fragment Analysis on CEQ8000

Samples were prepared according to the manufacturers protocol with 1µl of the PCR product and 4µl CEQ 400bp size standard in 40µl CEQ buffer/well of a 96-well CEQ sample plate. Oil was added to the wells to prevent evaporation during the denaturation step. Sample denaturation, electrophoresis, and fragment analysis were all incorporated in the CEQ 8000 "run" program.

CHAPTER 3

In Vitro Dendritic Cell Maturation

3.1 Introduction

3.1.1 Antigen Presenting Cells in Antigen-Driven T Cell Culture for Cellular Therapy

Successful *in vitro* expansion of CMV-specific T cell lines does depend to a large degree on the efficiency of antigen presentation by APC. Because of their key role, the choice of APC used in an *in vitro* culture system has a great influence on the quality and quantity of the culture output. Any manipulation of cultures carries a risk of infection, or induction of T cell responses to irrelevant antigens, and should be kept to a minimum. The ideal APC used for the *in vitro* activation of donor T cells would be readily available, and capable of effective processing and presenting of antigen, activating both CD4+ helper T cell and CD8+ CTL responses. There should be no non-specific activation of T lymphocytes.

3.1.2 Types of Professional APC

Blood APC include B cells, monocytes, macrophages, and DC precursors. The use of monocytes and B cells in an *in vitro* culture system would have the advantage of cutting out any further isolation steps, allowing for the antigen to be added directly to PBMC. This method is already being successfully utilised for CMV immunotherapy[45].

However, DC are the most potent APC and have been shown to induce much greater *in vitro* CMV-specific T cell proliferation than monocytes [56]. DC occur in blood only at very low frequencies (0.1%) [89]. In order to obtain DC in sufficient numbers, bone marrow- or peripheral blood-derived haemopoietic stem cells [90-93] or peripheral blood Mo [94-96] can be differentiated *in vitro* into DC. An advantage of BM-derived DC is their proliferative potential, but they tend to be less homogeneous in phenotype and function than MoDC [89]. Furthermore, BM would have to be collected from the donor as starting material.

3.1.3 MoDC

MoDC have been chosen as APC for this culture system, because T cells and monocytes can be obtained from the same single blood draw. Monocytes can be obtained from PBMC by positive selection of CD14+ cells with microbeads, by negative selection, or by adherence to plastic. Even though selection by adherence does not result in a highly pure population of Mo, it involves the least amount of manipulation and no extra reagents are required. The adherent fraction of PBMC is cultured in media containing GM-CSF and IL-4 for seven days, during which time Mo differentiate into DC. The DC generated in this way are not terminally differentiated. DC readily revert into macrophages upon growth factor withdrawal[94;95;97;98].

3.1.4 Maturation of MoDC

Immature DC take up and process antigen very efficiently, mainly by macropinocytosis [99], but also by phagocytosis, and through specialised receptors [89;100]. To become terminally committed, DC have to undergo a further maturation step. Once terminally matured their capacity for antigen uptake and processing is greatly reduced[89], at the same time they are most effective at presenting antigen to T cells.
In vivo, the interaction of immature antigen-presenting DC with individual T cells at the molecular level through TCR-MHC and accessory molecule engagement in combination with environmental factors and cytokines produced by DC and T cells, can induce final DC maturation.

In vitro induction of DC maturation has already received much attention by investigators. Conditioned media, cytokines, chemokines, and bacterial products, have all been shown in the literature to induce DC maturation (table 3.1). However, doubt has been cast on whether many of them were capable of inducing terminal, irreversible maturity[101;102]. Apart from bacterial LPS, monocyte-conditioned medium was the first agent used for the *in vitro* maturation of DC[95;97], but is impossible to standardise. Several authors have attempted to identify the cytokines and chemokines responsible for the DC-maturing effect of Mo-conditioned media. This resulted in the evaluation of cytokine cocktails, such as TNF-α and PGE_2[103], or TNF-α, IL-1, IL-6, and PGE2[97;104].

Based on the induction of DC maturation through the interaction of DC with T cells at the molecular level, soluble T cell surface molecules, such as CD154 (CD40L) have been tested as DC maturation agents [105;106]. The effect of soluble CD40L on DC maturation is well documented [80;103;106-108]. CD40L has also been combined with LIGHT [109], a member of the TNF superfamily.

Mature DC are unique in their ability to induce activation of naïve T cells. Finding a maturation signal which reliably produces a homogeneous population of mature DC may make it possible to activate CMV-specific T cells in CMV seronegative individuals, which has so far proven difficult in this system.

3.1.5 Study Objectives

Various mediators were compared here for their effect on DC function as antigen presenting cells in the culture system used for the expansion of CMV-specific T cells from CMV seropositive donors[56]. The aim was to identify a method for the optimisation of CMV antigen presentation, thus making the CMV culture system more robust and reliable, and to obtain CMV-specific T cells more rapidly.

Table 3.1 Publications on DC maturation

References	Maturation Factors	Tests	Result
Bender et al., 1996[94]	MCM compared to IL-1, IL-6, TNF-α, IL-12, IL-15	MLR, T cell proliferation, cytotoxicity assay (influenza)	Attempt to identify cytokines in MCM. MCM better than cytokine combinations. 1% plasma better than 10%
Rieser *et al.*, 1997 [110]	TNF-α (1000 U/ml) and PGE_2 (1 μM) compared to LPS [illegible] for 48h	IL-12 ELISA, FTSE-dextran uptake MLR	TNF-α and PGE_2 synergise, induction of mature phenotype (CD83), IL-12 production, allogeneic T cell proliferation
Jonuleit *et al.*, 1997 [104]	IL-1, IL-6, TNF-α, +/-PGE_2 compared to MCM, no FCS	T cell γ-IFN and IL-4 secretion assay, DC migration	MCM-mimic cocktail more effective than MCM, PGE_2 enhances DC maturation further. Increased IFN-γ production by T cells
Reddy *et al.*, 1997[97]	MCM compared to MCM-mimic cocktail of TNFα, IL-1beta, IFN-α	Immunophenotype, MLR	MCM more effective than cytokines
Snijders *et al.*, 1998 [111]	CD40L and IFN-γ	Cytokine ELISAs	CD40L and IFN-γ are both required for the induction of IL-12 production by IL-12, but both can be replaced by LPS
Kalinski *et al.*, 1998 [112]	TNF-α, IL-1β, or LPS +/- PGE_2 [illegible] for 48h	Immunophenotype, naïve T cell proliferation in response to SEB, IL-12 ELISA	PGE synergises with TNF-α and IL-1β: Phenotypic maturation, increased naïve T cell proliferation, but reduced IL-12 production, no effect on already mature DC.
Kalinski *et al.*, 1999 [113]	TNF-α (50 ng/ml) and IL-1β [illegible] for 48h +/- CD40L, IFN-γ, or bacteria	Immunophenotype, Cytokine ELISAs	Mature DC are unresponsive to IL-12 inducing stimuli, such as CD40L, IFN-γ, or bacteria. These are effective only at the onset of maturation.
Nelson et al. 1999 [101]	TNF-α [illegible] for 48h	Immunophenotype, MLR, KLH-specific T cell proliferation, inflammatory cytokine RT PCR	DC 'maturation' induced by TNF-α is reversible
Kuniyoshi *et al.*, 1999 [105]	CD40L+/- γ-IFN [illegible]	No immunophenotyping. T cell proliferation in response to tumor and Flu antigen, cytotoxicity assay, ELISPOT	CD40L+γIFN works best, then CD40L alone for induction of T cell proliferation, γ-IFN-production, specific lysis . IL15, but no IL-12 production by DC.
Ahonen *et al.*, 1999 [114]	R-848 [illegible] compared to LPS	Immunophenotype, MLR, Tet stain, toxicity and proliferation assays (antigen added with maturation factor), Cytokine/Chemokine ELISA	Similar DC maturation achieved with both
Schlienger *et al.*, 2000 [79]	TNF-α [illegible] for 4h)	Immunophenotype, FTSE-DX uptake, IL-12 ELISA, T cell proliferation assays: MLR, Tetanus, KLH	Induction of primary T cell response to KLH, IL-12 production by DC after contact with T cells.
Wutzen *et al.*, 2001 [106]	CD40L-Trimer [illegible], TNF-α [illegible] for 72h)	Immunophenotype CTL induction and cytotoxicity testing, cytokine production, allo-T cell-proliferation, PPD-specific proliferation	Increased surface marker expression, IL-12 production with CD40L, but no difference in CTL induction
Wesa & Galy, 2001 [107]	TNF, IL-1β, CD40L [illegible] (time of exposure not specified)	Immunophenotype, IL-12 secretion (ELISA and intracellular), no functional assays with T cells	IL-1β synergises with CD40L –induction of Il-12 secretion by T cells
Ebner *et al.*, 2001 [115]	CD40L, IFN-γ, IL-4	Immunophenotype, ELISA, intracellular cytokine secretion flow cytometry	IL-12-production greater in activated (with the various stimuli), immature DC than mature DC. MCM induces maturation (phenotypic) without IL-12 production
Morel *et al.*, 2001 [109]	LIGHT +/- CD40L [illegible] for 2d	Immunophenotype, macropinocytosis, MLR, Tumour ag cytotox. Assay, Cytokine ELISA	LIGHT augments CD40L-mediated induction of DC maturation. Enhanced IL-12, TNF-α and IL-6 production by DC, enhanced performance in MLRs CTL-induction, IFN-γ secretion by CTL
Lee *et al.*, 2002 [80]	IL-1, IL-6, TNF-α, PGE_2 cocktail compared to dsRNA, MCM, CD40L	MLR, ELISPOT with KLH and Flu, cytokine ELISA,	Primary T cell response, mature phenotype, no IL-12 production with cocktail
Luft et al., 2002 [103]	TNF-α, IFN-α, CD40L, all +/-PGE_2	Transwell migration, MLR, Cytokine ELISA	PGE_2 switches on migratory function, other cytokines switch on cytokine production
Luft *et al.*, 2002 [116]	CD40L [illegible]+/-IFN-α (100 U/ml) [illegible] for 2d	Immunophenotype, transwell migration, MLR, Cytokine ELISA, ELIspot	IFN-α enhances CD40L-induced DC maturation. Enhanced IL-12, IL-10 and IL-6 production by DC, enhanced induction of IFN-γ secretion by allo- T cells.
Luft *et al.*, 2002 [117]	CD40L [illegible]+/- IL-β (1-2ng/ml) [illegible] for 72h	Transwell migration, MLR, Cytokine ELISA	IL-1β enhances CD40L-induced DC maturation. Enhanced IL-12, IL-10 and IL-6 production by DC, enhanced induction of IFN-γ secretion by allo- T cells.
Buchler *et al.*, 2003 [118]	1 step: cytokines added at the same time. (TNF-α, IL1α, IL-6, PGE1) (GM-CSF, PGE1), (GM-CSF PGE1, IFN-γ)	Phenotype, yield, no T cell tests	Emphasis on yield: (GM-CSF PGE1, IFN-γ) gave highest no of CD83+. Low HLA DR expression throughout
Nagai *et al.*, 2003 [119]	TNF-α in combination with IFN-β	In vitro model of human MoDC-dependent, superantigen-mediated, naïve T cell differentiation	IFN-β augments TNF-α-mediated DC maturation, induction of IFN-γ-secreting TH1 cells.

3.2 Special Methods

3.2.1 Evaluation of DC Maturation: Immunophenotyping

Naturally occurring DC originate from various myeloid and lymphoid precursors. Single surface markers that define DC have not been identified, but DC are characterised by extremely high levels of the molecules involved in antigen presentation to T cells[89].

MoDC were generated as described in Chapter 2. Differentiating MoDC were exposed to various mediators of DC maturation. Resulting MoDC populations were evaluated by flow cytometry, staining for surface molecules associated with antigen presentation: HLA DP, DQ, DR, and accessory molecules for antigen presentation: CD86, CD40. The surface molecule CD83 served as a marker for DC maturation[120], its precise function is currently unknown [121;122]. Loss of the monocyte-specific marker CD14 was also verified. The frequencies of cells binding surface marker-specific mabs above the isotype controls, and MFI of surface marker positive fractions were measured by flow cytometry. The MFI allows a comparison of the mean brightness of staining of marker positive cells, providing a way of comparing levels of surface molecule expression/cell in different populations. However, MFI is dependent on staining conditions and laser power, hence is only useful for relative comparisons between cell populations stained at the same time and analysed in the same flow cytometry session.

Adherent Mo were obtained from 2-6 million PBMC in 6- or 12 well plates, and differentiated into MoDC with 100ng/ml GM-CSF and 100ng/ml IL-4 for typically 7 days. GM-CSF and IL-4 were replenished on day 3 or 4 of culture. Differentiating MoDC were pulsed with CMV- or control antigen on day 3. Various candidate cytokines, cytokine cocktails, or other agents for DC maturation were added between days 4 and 6.

3.2.2 Evaluation of DC Function: T Cell Proliferation Assays

Assays measuring induction of antigen-specific T cell proliferation (see chapter 2: General Methods) have the advantage over MLRs of providing a measure of MoDC's ability to induce T cell proliferation specific for an antigen of interest. The capacity of various DC populations to present CMV antigen to T cells was evaluated. For this purpose MoDC were first washed to remove any added cytokines.

Proliferation assays were set up essentially as described in General Methods (section 2.8), plating 3000 to 10000 DC/well with 100 000- 200 000 autologous PBL/well. Cell numbers were constant throughout each assay, to enable the comparison of responses at a specific time point within assays. Cell numbers were variable across different assays, so direct inter-assay comparisons were not permissible.

The CMV antigen is a whole cell lysate, derived from a human lung fibroblast cell line infected with the laboratory strain AD169 of CMV. The preparation contains non-CMV components as well as CMV antigens. T cell responses to non-CMV antigen were dissociated from CMV-specific responses by inclusion of a control antigen derived from the same, but uninfected lung fibroblast cell line into the proliferation assay set-up. T cell proliferation in response to control antigen may indicate the activation of alloreactive T cells. These may increase the risk of GvHD to patients potentially receiving this cell product. Therefore APC used in the culture system are required to induce high levels of CMV-specific T cell proliferation, but at the same time minimum levels of proliferation in response to the control antigen.

3.2.3 Dosing and Timing of Exposure to Maturation Factors

Conditions for DC maturation with various mediators were initially taken from the published literature and modified where required. For example, other investigators added TNF-α to differentiating MoDC at various time points between days 0 and 12 of MoDC differentiation[79;101;110;118]. Here, following the published method by Schlienger et al. [79], TNF-α was added at a concentration of 20ng/ml to MoDC on days 4 and 6 of differentiation. Immature DC take up antigen from their environment most effectively, hence CMV or control antigens were added at least 3h before the addition of any

maturation factors to allow maximum antigen uptake. Concentration and timing of TNF-α exposure were then optimised as described in Results.

Several groups used TNF-α in combination with PGE_2 for the induction of DC maturation [103;110], adding these factors to differentiating MoDC at various time points between days 5 and 10. Here, according to the method described by Luft *et al.*[103], Mo were allowed to differentiate into immature DC for 7 days, before being exposed to the maturation cytokines.

Concentration and timing of addition of the components of the MCM-mimic cocktail were taken from the literature [97;103], and modified for some components, according to optimisation experiments described below. TNF-α (20-100ng/ml), IL-1 (10ng/ml), IL-6 (10ng/ml) were added to differentiating MoDC on day 4, PGE_2 (1μg/ml) was added on day 6 of the 7-day differentiation.

Addition of CD40L was optimised by titration and timing experiments. The same titration was applied to LIGHT for the dose optimisation.

3.3 Results

3.3.1 Phenotype of CMV Antigen-Pulsed MoDC

Since the purpose of this work was to compare phenotype and function of MoDC treated with various maturation agents to untreated MoDC, the normal variability of surface marker expression by CMV antigen-pulsed MoDC was established first.

Frequencies of DC expressing the various surface markers are shown in figure 3.1 for up to eight donors. The median percentage of CD83-expressing DC in CMV antigen-pulsed MoDC from eight different donors was 14.5% (4-46%) (Figure 3.1A). In CMV-antigen-pulsed MoDC generated from a single donor on four separate occasions, CD83 expression varied between 12-30% (median 16%) (data not shown). Frequencies of cells expressing CD40, CD86, HLA class II were high (>70%) in all donors and all experiments (Figure 3.1B, C, D), with the exception of one experiment in which only 40% of MoDC expressed CD86 (Figure 3.1C). Due to the relatively great variability of CD83 expression direct comparisons were only made between normalised data, or between MoDC populations

generated at the same time from the same blood sample, whilst avoiding direct comparisons across different batches of MoDC.

3.3.2 TNF-α as a DC Maturation Agent

3.3.2.1 TNF-α-Treated MoDC Induce Variable Levels of Autologous CMV-Specific T Cell Proliferation

DC from three CMV seropositive donors differentiated *in vitro* from monocytes in eight days with GM-CSF and IL-4, and exposed to 20ng/ml TNF-α on the fourth and the sixth day of their differentiation induced more autologous T cell proliferation than untreated DC in response to CMV antigen in only 2/4 assays (Figure 3.2A and B). Additionally, in one these two assays, there was increased T cell proliferation in response to control antigen as well as CMV antigen (Figure 3.2B), making the T cell response of TNF-α-treated DC less CMV antigen-specific compared to untreated MoDC. In the other two experiments using cells from a single donor, TNF-α-treated MoDC induced similar levels of CMV-specific T cell proliferation to untreated DC (Figure 3.2C and D).

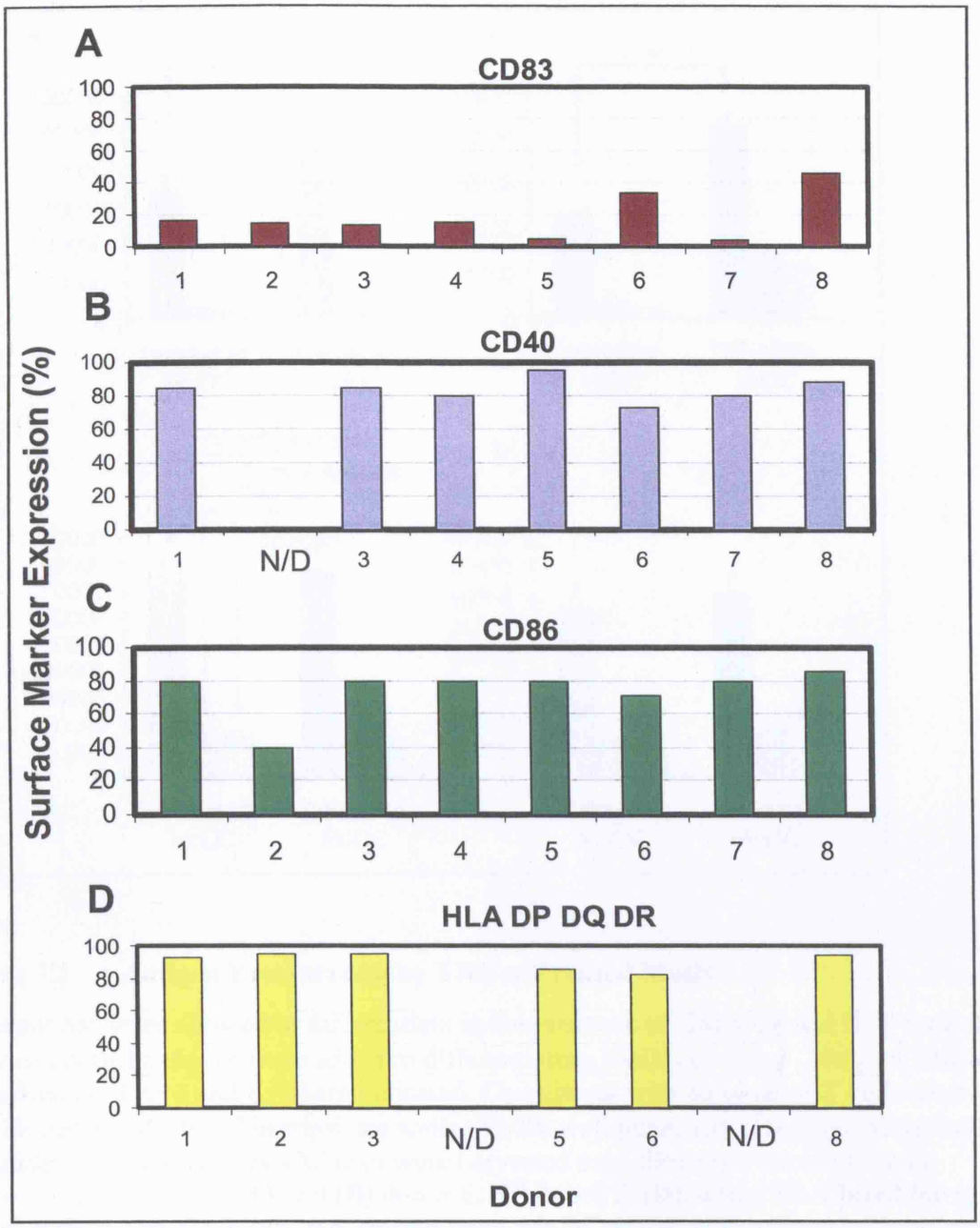

Figure 3.1 Surface Marker Expression by Immature MoDC

Adherent or CD14-selected monocytes from up to 8 different donors were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 for 7 days. On day 3, differentiating MoDC were pulsed with CMV antigen. On day 7, MoDC were stained with anti CD83, CD40, CD86, HLA DP DQ DR, or isotype control mabs conjugated to FITC or PE, and analysed by flow cytometry. Graphs show frequencies of surface marker positive cells. **(N/D)** not done.

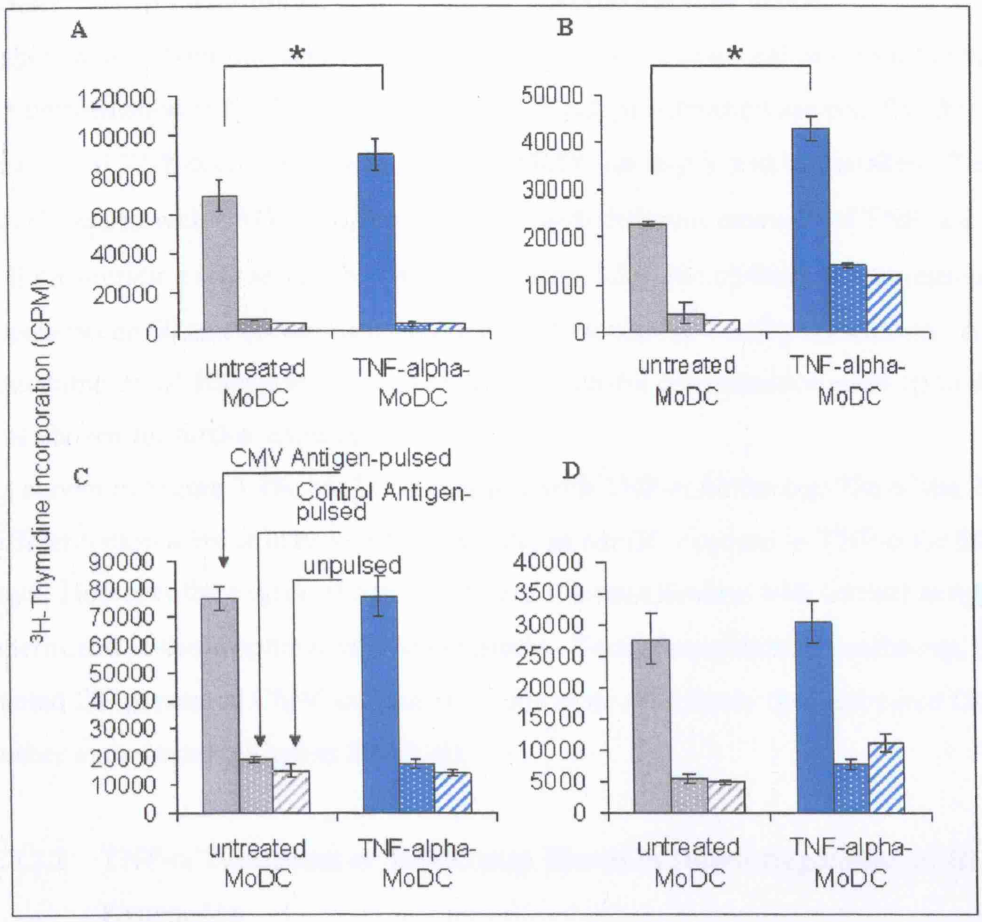

Figure 3.2 Antigen Presentation by TNF-α-Treated MoDC

Adherent Mo were allowed to differentiate in the presence of GM-CSF and IL-4 for 8 days. CMV or control antigens were added to differentiating MoDC on day 3. 20ng/ml TNF-α was added on days 4 and 6, where indicated. Co-cultures with autologous T cells were set up with irradiated MoDC in triplicate wells of a 96-well plate. 1μCi ^{3}H-Thymidine/well was added for the final 16h. Cultures were harvested onto filtermats for scintillation counting on day 5 or 6. **(A)** and **(B)** donor 6, **(C)** donor 9; **(D)**, donor 10. **Closed bars,** MoDC pulsed with CMV antigen; **dotted bars,** MoDC pulsed with control antigen; **striped bars,** MoDC pulsed with no antigen; **error bars,** standard deviations of mean of triplicate cultures; **asterisks**, significant difference.

3.3.2.2 Optimisation of TNF-α-Induced Maturation of MoDC

Exposure to suboptimal concentrations of TNF-α was a potential cause for the variability in the performance of TNF-α-treated MoDC in T cell proliferation assays. For this reason, a titration of TNF-α concentration, added to MoDC on day 5, was undertaken. The capacity of DC pulsed with CMV antigen and treated with different amounts of TNF-α to induce T cell proliferation was tested. As shown in Figure 3.3A, the optimal concentration of TNF-α was between 60 and 180ng/ml for induction of maximum T cell proliferation. A concentration of 100ng/ml, five times greater than the concentration used up to this point, was chosen for further experiments.

As shown in Figure 3.3B, MoDC stimulated with TNF-α for the last 72h of the 7-day-differentiation were at least as effective APC as MoDC exposed to TNF-α for the whole 7 days. However the degree of non-CMV-specific proliferation with control antigen was not determined in these optimisation experiments. Using the optimised conditions, TNF-α-treated DC presented CMV antigen to T cells more effectively than untreated DC in 2/2 further experiments (Figures 3.14A/B).

3.3.2.3 TNF-α Treatment of MoDC may Result in Down-Regulation of HLA Class II Expression

Immunophenotypes of MoDC, untreated, or matured with TNF-α, are summarised in table 3.2. They differed in several respects. In a series of seven experiments, the maturation marker CD83 was expressed by a significantly higher percentage of TNF-α-treated MoDC than by untreated MoDC (paired t-test, 6 df, $p=0.042$). In 4/7 experiments with cells from different donors, at least twice as many TNF-α-treated MoDC (median 31%, range: 29-47%) than untreated MoDC (median 14.5%, range 1-15%) expressed the DC-maturation marker CD83. Percentages of CD83 positive DC from the other 3/7 donors were similar, irrespective of TNF-α treatment.

The MFI of the CD83-positive fractions was markedly elevated in MoDC treated with TNF-α compared to untreated MoDC in just 2/7 experiments, as shown in table 3.2. In the other 5 experiments, the average brightness of fluorescence staining/cell was similar in both MoDC populations, indicating expression of similar numbers CD83 molecules per cell.

Overall, there was no significant difference between the MFI of the CD83 positive fractions between TNF-treated and untreated MoDC.

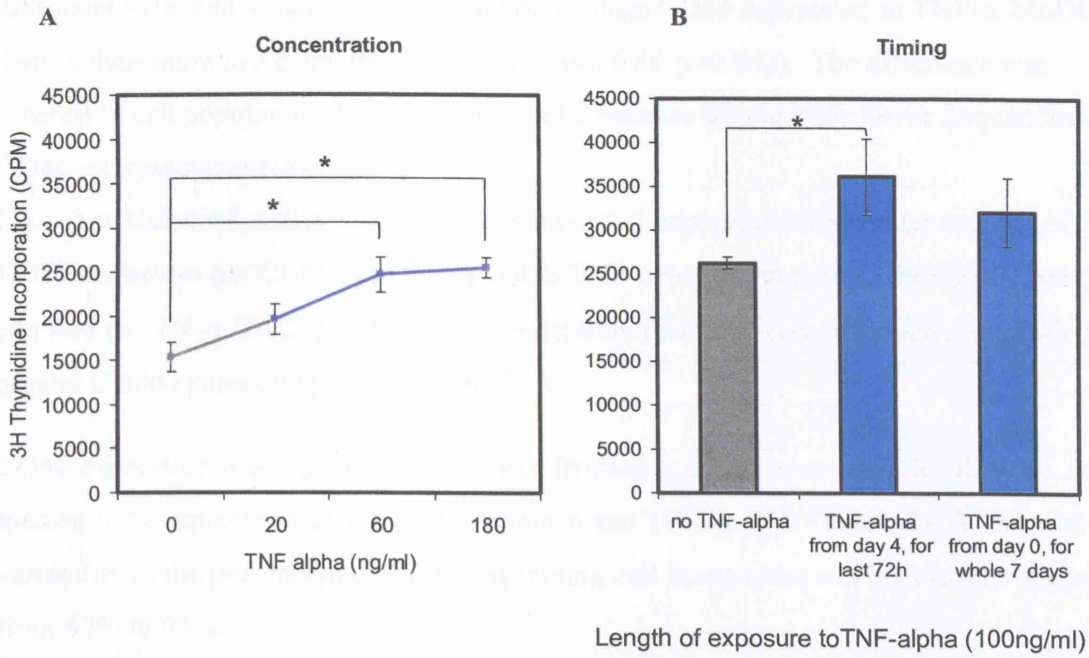

Figure 3.3 Antigen Presentation/T Cell Proliferation Assay - Optimisation of TNF-α-Mediated DC Maturation

A, MoDC from CMV seropositive donor 6 were pulsed with CMV antigen before being treated with a range of concentrations of TNF-α on day 4 of their 7-day differentiation. They were irradiated and tested as inducers of antigen-specific T cell proliferation in co-cultures with autologous T cells in triplicate wells of a 96-well plate. 1μCi ^{3}H-Thymidine/well was added 16h before harvesting the cultures for scintillation counting on day 4. **B**, MoDC from CMV seropositive donor 1 were exposed to TNF-α (100ng/ml) for the whole 7 days of their differentiation, for the last 72h, or differentiated without TNF-α, after pulsing with CMV antigen. They were irradiated and tested as inducers of autologous T cell proliferation in 5-day triplicate co-cultures. 1μCi ^{3}H-Thymidine/well was added 16h before harvesting the cultures. Tritiated Thymidine incorporation into nucleic acids was determined by scintillation counting. **Grey bars**, no TNF-α; **blue bars** TNF-α; **Error bars**, standard deviation of means of triplicate wells.

Although percentages of cells expressing CD86 were high in TNF-α-MoDC (median 84%; 73-93%) as well as in untreated MoDC (median 80%; 33-86%), statistical analysis of seven data pairs indicated a significantly higher percentage CD86 expression in TNF-α-MoDC than in their untreated counterparts (paired t-test, 6 df, $p=0.042$). The difference was greatest in cell populations from donors 1 and 2 because of relatively lower frequencies of CD86-expressing untreated DC (33-38%).
Likewise, statistical analysis of MFI ratios indicated expression of a greater number of CD86 molecules per CD86-expressing cell in TNF-α-MoDC than in CD86+MoDC not exposed to TNF-α ($0.02<p<0.05$t, 6d.f.), indicating that TNF-α may induce a somewhat greater CD86 expression per cell (Table 3.2).

CD40 expression was high, both in terms of frequency and number of molecules/cell. A median 87% expressed CD40 in both untreated and TNF-α-DC (Table 3.2). There was variability in the percentage of CD40-expressing cell frequencies at a high level, ranging from 67% to 95%.

More than 90% of MoDC expressed HLA DP DQ DR in both untreated and TNF-α-treated MoDC alike (6/6 experiments). However, in 4/6 experiments MoDC treated with TNF-α expressed a reduced number of HLA class II molecules per cell, compared to untreated MoDC, i.e. the MFI of the HLA positive fraction of TNF-α -treated MoDC was reduced by 20-60% compared to untreated MoDC. In the other 2 experiments, the MFI values were similar for untreated and TNF-α treated MoDC (Table 3.2). However, more experiments are required to determine the significance of this observation, since the p-value generated by the t-test (5 df) was above 0.05.

The number of experiments was too small to be able to comment on a possible difference in phenotype between MoDC matured with TNF-α at 20ng/ml compared to 100ng/ml.

A) Frequency of Surface Marker Expression

Donor	CD83 *p=0.04		CD40 p=0.90		CD86 *p=0.04		HLA II p=0.44	
	MoDC (%)	MoDC-TNF (%)	MoDC (%)	MoDC-TNF (%)	MoDC (%)	MoDC-TNF (%)	MoDC (%)	MoDC-TNF (%)
20ng/ml TNF-α (added on days 3 and 5)								
1	13	47	N/D	N/D	33	73	93	94
2	15	31	69	84	38	74	97	97
3	12	32	90	92	85	89	98	92
4	16	17	86	67	80	84	N/D	N/D
100ng/ml TNF-α (added on day 4)								
6	1	29	95	93	89	94	85	90
7	33	36	71	76	71	82	82	92
9	46	42	88	91	86	93	94	95

B) Mean Surface Marker Fluorescence

Donor	CD83 p=0.077			CD40 p=0.509			CD86 *p=0.042			HLA II p=0.079		
	MFI: MoDC	MFI: MoDC-TNF	MFI Ratio: MoDC-TNF/ MoDC	MFI: MoDC	MFI: MoDC-TNF	MFI Ratio: MoDC-TNF/ MoDC	MFI: MoDC	MFI: MoDC-TNF	MFI Ratio: MoDC-TNF/ MoDC	MFI: MoDC	MFI: MoDC-TNF	MFI Ratio: MoDC-TNF/ MoDC
20ng/ml TNF-α (added on days 3 and 5)												
1	1	1	1	N/D	N/D	N/D	28	34	1.21	78	21	0.27
2	1	1	1	16	18	1.13	26	34	1.31	45	21	0.47
3	4	5	1.25	22	26	1.18	15	32	2.13	98	60	0.61
4	2.5	2.6	1.05	10	6	0.6	13	14	1.07	N/D	N/D	N/D
100ng/ml TNF-α (added on day 4)												
6	13	22	1.69	57	70	1.23	25	92	3.68	81	89	1.10
7	6.5	12	1.84	13	17	1.31	67	85	1.27	147	151	1.03
9	4	4.5	1.13	21	21	1	42	42	1	42	33	0.78

Table 3.2 Surface Marker Expression by MoDC +/- TNF-α

20ng/ml, or 100ng/ml TNF-α were added to differentiating MoDC on days 4 and 6 of their 7-day differentiation as indicated. On day 7, DC were single or double stained with anti-CD83-FITC, CD40-PE, CD86-PE, HLA DP, DQ, DR-FITC and analysed by flow cytometry. The table compares **(A)** DC treated with TNF-α to DC not exposed to added TNF-α with respect to surface marker expression frequencies (%)and **(B)** MFI. To allow comparisons between different experiments, the MFI of untreated MoDC was normalised to 1.

*, significant differences between TNF-α-MoDC and their untreated counterparts in expression frequencies (paired t-test, n-1 df), or in MFI (t-test, N.H.: mean of ratios= 1, n-1 df). p-values shown in brackets.

3.3.3 TNF-α and PGE_2

3.3.3.1 TNF-α and PGE_2-Treated MoDC were Mature by Morphology and Phenotype

Adding TNF-α (20ng/ml), PGE_2 (5μM) to differentiating MoDC on day 7 for the final 48h of their differentiation with GM-CSF and IL-4 resulted in a more mature morphological appearance when compared to the same DC not exposed to TNF-α and PGE_2 (Figure 3.4A), with large cytoplasmic extensions in the shape of dendrites and veils.

Immunophenotyping cells from three donors showed that the proportion of MoDC expressing CD83 exceeded 60% (up to 90%) following treatment with TNF-α+PGE_2, compared to between 5% and 46% in untreated MoDC, respectively (Figure 3.5A). Mean expression levels of CD83 per CD83-positive DC increased by 20-100% with exposure to TNF-α and PGE_2 in all three donors (Figure 3.5B). These data indicate TNF-α and PGE_2-induced expression of mature-type surface markers.

Frequencies of CD40 and CD86-expressing MoDC were high (>/=80%) in untreated- and TNF-α+PGE_2-treated MoDC alike. However, there was a difference in the amount of CD40 or CD86 surface molecules expressed per marker-positive cell; the MFI was greater for MoDC treated with TNF-α and PGE_2 than for untreated MoDC. CD86 expression/cell increased more than CD40 expression/cell.

The frequency of HLA class II-expression exceeded 90% in MoDC treated with TNF-α and PGE_2 and untreated MoDC alike (Figure 3.5A, Donors 1 and 8).

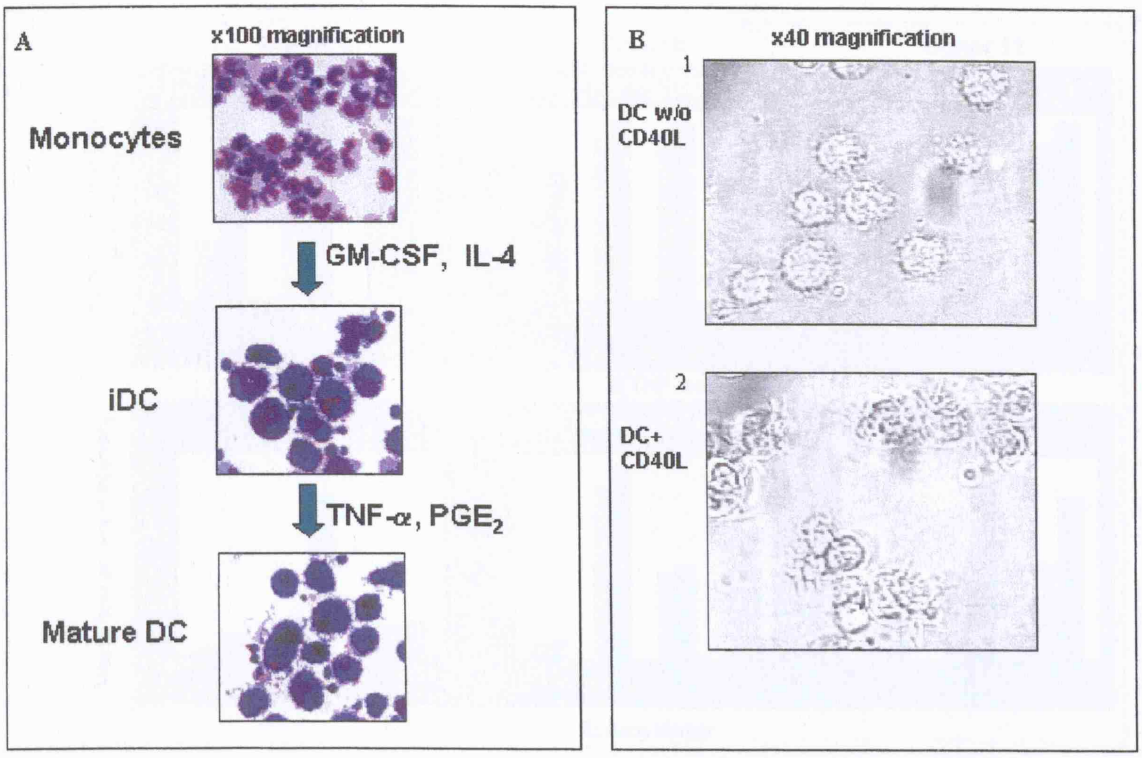

Figure 3.4 Light micrographs of MoDC

A, MGG-stained cytospins. Monocytes, MoDC differentiated from Mo in the presence of GMCSF and IL-4, and mature MoDC exposed to 20ng/ml TNF-a and 5μM PGE2 from day 7 for 48h. **B,** live MoDC in culture; **B1**, untreated day 8 MoDC; **B2**, MoDC treated with CD40L (300ng/ml) for the last 48h of the 8-day differentiation with GM-CSF and IL-4.

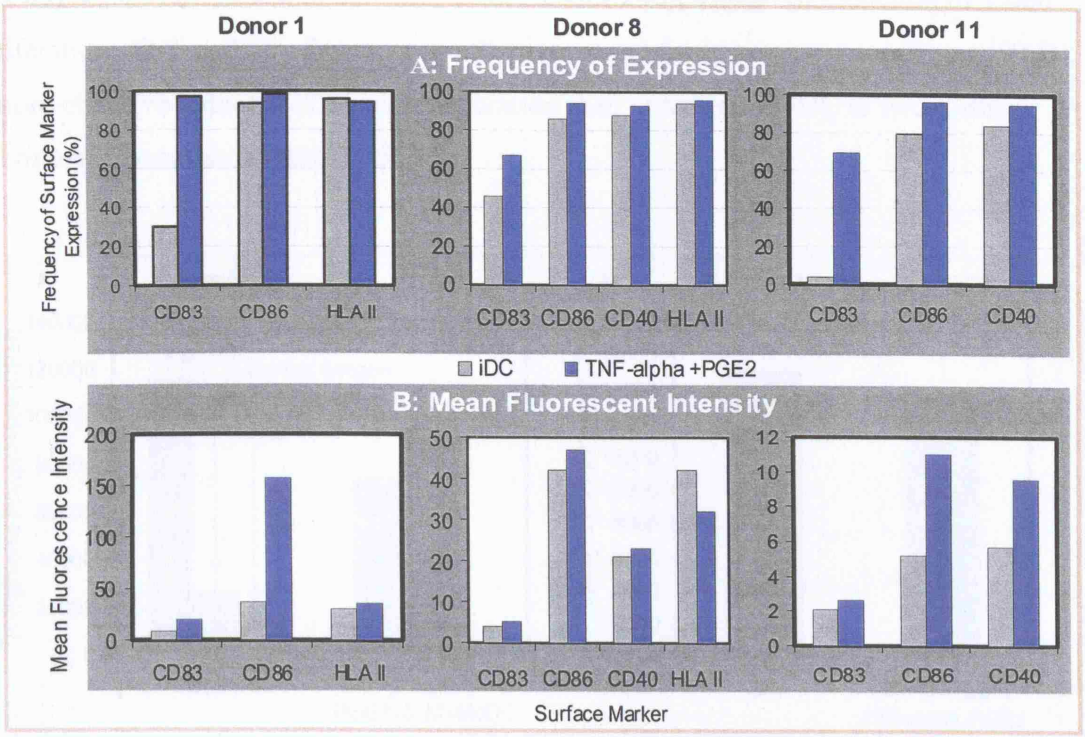

Figure 3.5 Surface Marker Expression by MoDC Matured with TNF-α and PGE_2

MoDC were generated from three donors in the presence of GM-CSF and IL-4. Donors 1 and 11: adherent monocytes, donor 8: CD14-selected monocytes. Donor 11: 20ng/ml TNF-α and 5μM PGE2 were added on day 7 of differentiation for the next 48h. Donors 1 and 8: 100ng/ml TNF-α was added on day 4, 1μg/ml PGE2 was added on day 6 for 24h. On day 9 or 7, respectively, DC were stained with FITC- or PE-conjugated mab against CD40, CD86, CD83, HLA DP DQ DR, or isotype control mabs. **A**, Frequency of positive cells (%); **B**, MFI (arbitrary units); **grey bars**, untreated MoDC; **blue bars**, MoDC treated with TNF-α and PGE2.

3.3.3.2 MoDC Treated with TNF-α and PGE_2 were Poor Inducers of Antigen-Specific Autologous T Cell Proliferation

Despite this apparent increase in the frequency of mature DC as defined by CD83 expression, raised expression levels of co-stimulatory molecules, and 'mature'-type morphology, DC treated with TNF-α and PGE_2 presented CMV antigen to T cells with lower efficiency compared to untreated MoDC (Figure 3.6A). Adding TNF-α to differentiating MoDC at the optimal concentration determined in section 3.3.2.2 (100ng/ml

from day 4 for 72h), followed by PGE_2 on day 6 (for 24h), improved induction of T cell proliferation. TNF-α PGE_2-DC were as effective in one further experiment (Figure 3.6B), or more effective inducers of T cell proliferation than untreated MoDC in two further experiments (example: Figure 3.15C).

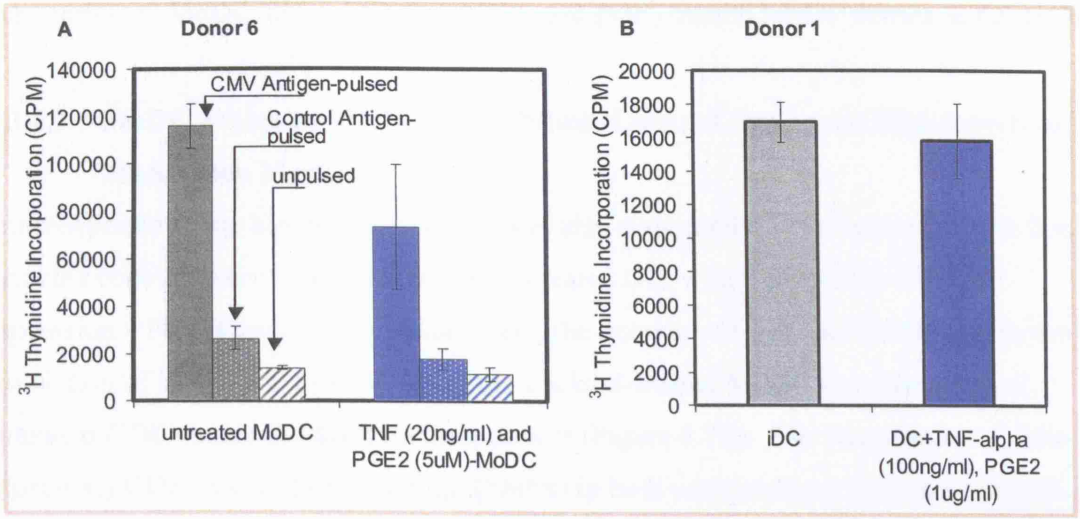

Figure 3.6 Antigen Presentation/ T Cell Proliferation Assay – Induction of Autologous Antigen-Specific T Cell Proliferation by MoDC Treated with TNF-α and PGE_2

A, MoDC from CMV seropositive donor 6, pulsed with no antigen **(striped bars)**, control antigen **(dotted bars)**, or CMV antigen **(closed bars)**, were matured with 20ng/ml TNF-α and 5μM PGE2 **(blue)** on day 7 of their 9-day differentiation, or left untreated **(grey)**. **B,** Monocytes from CMV seropositive donor 1 were allowed to differentiate into MoDC for 7 days. CMV antigen and TNF-α (100 ng/ml) were added to differentiating MoDC on day 4. PGE_2 (1μg/ml) was added on day 6. For both donors, 10 000 irradiated MoDC were co-cultured in triplicate wells of a 96-well plate with 200 000 autologous PBL. Co-cultures were maintained for 6 days. 1μCi of ^{3}H-Thymidine/well was added for the last 16h of culture, before harvesting. CPM were determined by scintillation counting. **Error bars**, standard deviation of means of triplicate wells.

3.3.4 TNF-α, PGE_2, IL-1, and IL-6 (MCM-Mimic Cocktail)

3.3.4.1 MCM-Mimic Cocktail-Treated MoDC had 'Mature-Type' Morphology

Morphologically, the resulting day 7 MoDC appeared more mature than untreated MoDC. MoDC treated with the cytokine cocktail had greater numbers of larger dendrites and veils than untreated MoDC, much like the TNF-α and PGE_2 treated MoDC shown in figure 3.4.

3.3.4.2 MoDC Treated with the MCM-Mimic Cocktail Expressed High Levels of Maturation Markers

Immunophenotyping MoDC from two donors also suggested that those treated with this cytokine cocktail were more mature than untreated DC, with a frequency of CD83 expression >70% (Figure 3.7A). In addition, the number of CD83 molecules/cell in the population of CD83-positive MCM-mimic-cocktail-treated MoDC was twice that of untreated CD83-positive MoDC in both donors (Figure 3.7B). The frequencies of cells expressing CD86 and CD40 were high (>80%) in both untreated and cytokine-cocktail-treated MoDC populations, and the number of CD86 molecules/cell was up-regulated in cytokine-cocktail-treated CD86-positive MoDC by 200-500% in cells generated from both donors compared to untreated CD86-positive MoDC (Figure 3.7B). CD40 expression/cell was similar or slightly raised in cytokine-cocktail-treated/CD40 positive- compared to untreated CD40 positive MoDC (Figure 3.7B). These results were highly reproducible when MoDC were generated and analysed from the same donor on two separate occasions (Data not shown).

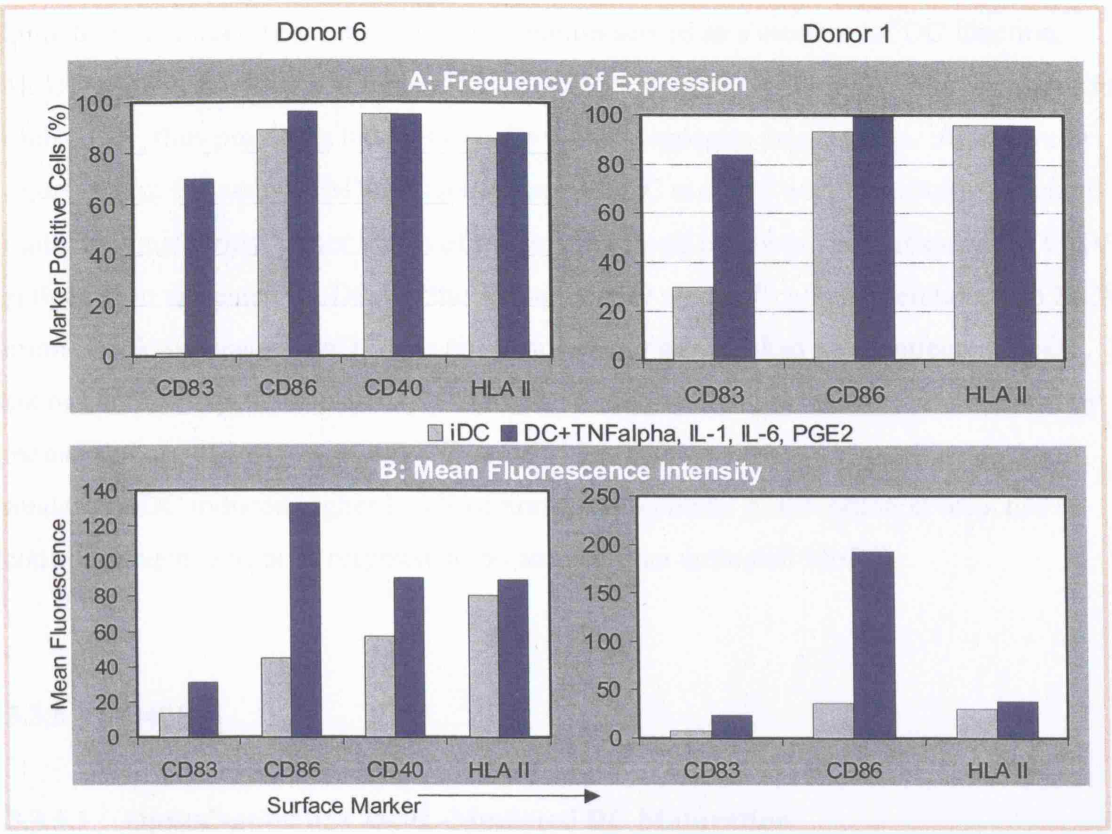

Figure 3.7 Effect of the MCM-Mimic Cocktail on MoDC Phenotype

Adherent Mo from two donors were differentiated into MoDC in the presence of GM-CSF and IL-4 for 7 days in 24-well plates and treated with a cocktail of TNF-α (100ng/ml), IL-1 (10ng/ml), IL-6 (10ng/ml), and PGE2 (1μg/ml), where indicated. TNF-α was added for the last 72h, the other agents for the last 24h. On day 7 MoDC were labelled with FITC or PE-conjugated isotype control, anti-CD83 –CD86 -CD40 or -HLA DP DQ DR mabs. The cells were then analysed by flow cytometry. **A**, % Frequency cells expressing surface markers; **B**, Mean surface marker expression/cell; **grey bars**, untreated MoDC; **blue bars**, MCM-mimic-cocktail-treated MoDC.

3.3.4.3 MoDC Matured with the MCM-Mimic Cocktail were Inducers of Non-Specific T Cell Proliferation

Induction of antigen-specific T cell proliferation served as a measure of DC function. MoDC were generated from three different CMV+ donors, on three separate occasions for each donor, thus providing batches of cells for nine separate experiments. As shown in figure 3.8, in 6/9 assays CMV antigen-pulsed MoDC matured with the cytokine cocktail induced significantly higher levels of autologous T cell proliferation in response to CMV antigen than untreated MoDC. In the remaining 3/9 assays T cell proliferation with MCM-mimic-cocktail-treated MoDC was not significantly greater than with untreated MoDC. In six of the nine experiments the specificity of T cell proliferation was also determined by including control antigen-pulsed DC as APC. In 4 of these 6 assays, cytokine cocktail-treated MoDC induced higher levels of non-CMV-specific proliferation in response to control antigen, and/or in response to no antigen than untreated MoDC.

3.3.5 CD40L

3.3.5.1 Optimisation of CD40L-Mediated DC Maturation

Recombinant human CD40L was assessed here as an inducer of DC maturation. Concentration (Figure 3.9A) and timing of CD40L-addition (Figure 3.9B) to differentiating MoDC were optimised in T cell-proliferation induction assays. As seen in figure 3.9, DC treated with 300ng/ml CD40L on day 4 or 5, for 48-72h, one day after addition of CMV antigen, achieved the highest levels of autologous T cell proliferation.

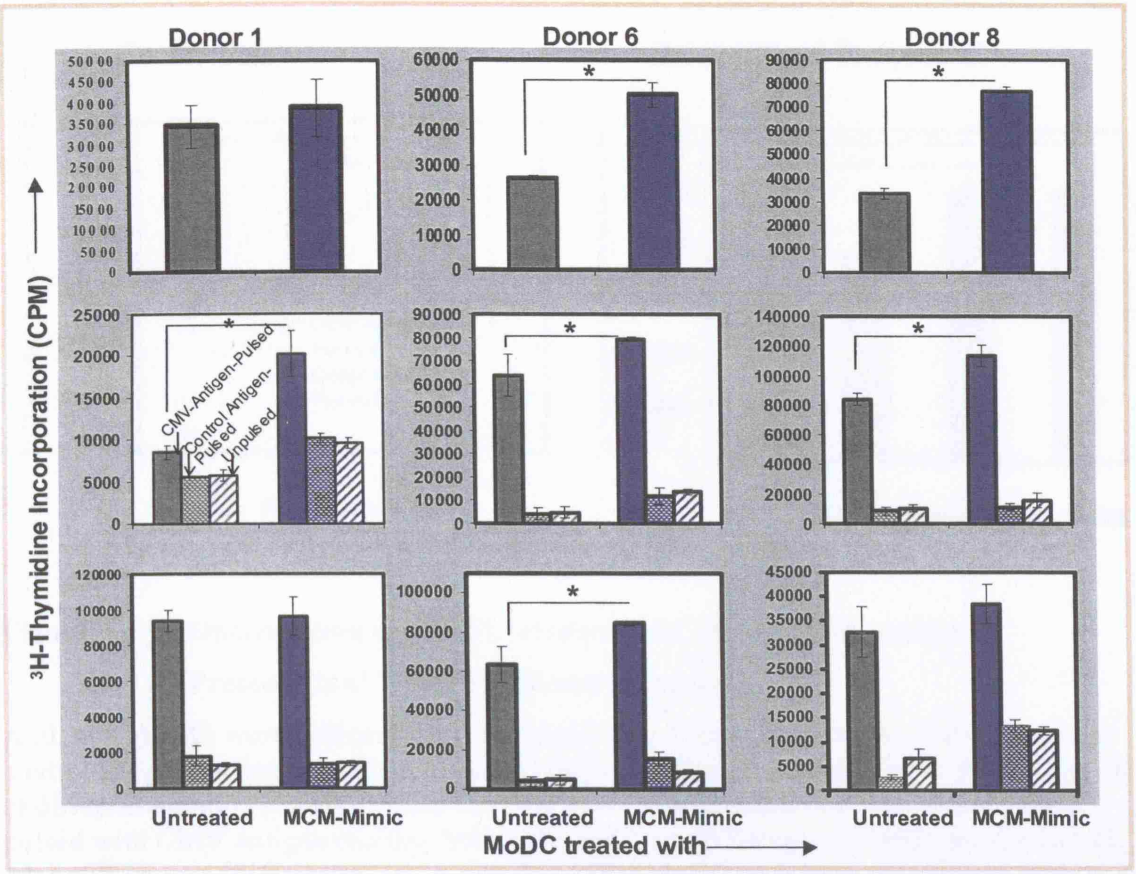

Figure 3.8 T cell proliferation induced by MoDC treated with a cocktail of TNF-α, IL-1, IL-6, and PGE2

Adherent Mo from three CMV seropositive donors were allowed to differentiate into MoDC in the presence of GM-CSF (100ng/ml) and IL-4 (100ng/ml) for 7 days. On day 4, the differentiating MoDC were pulsed with antigen and treated with TNF-α (100ng/ml) 3h later. On day 6, IL-1 (10ng/ml), IL-6 (10 ng/ml), and PGE_2 (1mg/ml) were added. On day 7, the cytokines were washed off and the MoDC were co-cultured with autologous PBL at a ratio of 1 : 10-20 in triplicate wells of 96-well plates. 1μCi ^{3}H-Thymidine/well was added on day 5 or 6 for the last 16h before harvesting. 3H-Thymidine incorporation was measured by scintillation counting. Results are displayed as and average of triplicate wells, showing mean CPM. **Error bars**, standard deviation of means of triplicates; **blue**, MCM-mimic-cocktail-treated MoDC; **grey**, untreated MoDC; **solid bars**, DC pulsed with CMV antigen; **dotted bars**, DC pulsed with control antigen; **diagonal lines**, DC pulsed with no antigen; **asterisks**, significant difference.

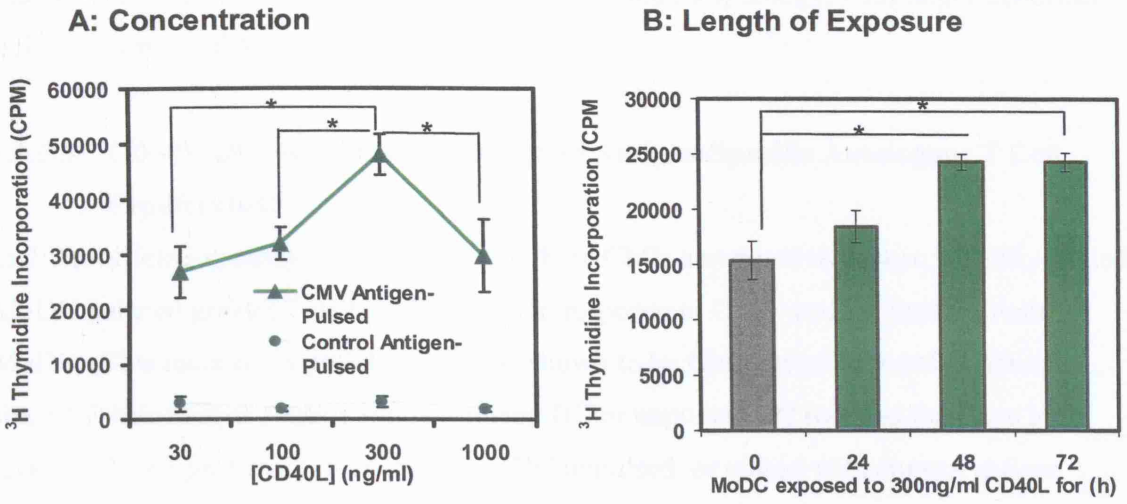

Figure 3.9 Optimisation of CD40L-Mediated DC-Maturation: Antigen Presentation/ T Cell Proliferation Assays.

A (donor 1), Mo were differentiated into MoDC for 7 days, pulsed with CMV antigen or control antigen on day 5, and treated with various concentrations of CD40L for the last 48h of differentiation. **B** (donor 6), adherent Mo were differentiated into MoDC for 7 days, pulsed with CMV antigen one day before the addition of 300ng/ml CD40L for the last 24, 48, or 72 hours of differentiation. **A** and **B**, 10 000 MoDC/well were co-cultured with 200 000 autologous PBL/well in triplicate wells of a 96-well plate. After 5 days of co-culture, in the presence of 1μCi of ^{3}H-Thymidine/well for the last 16h. Plates were harvested on day 6 and the counts/minute quantified by scintillation counting. **Green**, CMV antigen-pulsed TNF-α-MoDC; **blue-green**, control antigen-pulsed TNF-α MoDC; Grey, CMV-antigen-pulsed MoDC matured with TNF-α; **error bars**, standard deviation of the mean of triplicate values, **asterisks** indicate significant differences between the mean of triplicate values.

3.3.5.2 MoDC Treated with CD40L Had Mature-Type Morphology

The micrographs in figure 3.4B show morphological differences between untreated- and CD40L-DC. CD40L-MoDC displayed a more mature morphology, with larger dendrites and veils compared to untreated MoDC.

3.3.5.3 CD40L-DC were Potent Inducers of Antigen-Specific Autologous T Cell Proliferation

In 7/7 proliferation assays using cells from three CMV seropositive donors, CD40L-treated MoDC induced greater T cell proliferation in response to CMV antigen than untreated MoDC. This increase in proliferation was shown to be CMV-specific for all 3 donors, since CD40L-treated control antigen-pulsed DC or unpulsed DC induced the same low levels of T cell proliferation as immature DC unpulsed, or pulsed with control antigen (Figure 3.10).

3.3.5.4 Heterogeneous Surface Maturation Marker Expression by CD40L-DC

Immunophenotypes of CD40L-MoDC compared to untreated MoDC generated from two donors are summarised in table 3.3 (see also figure 3.16). There was a rise in the percentage of DC expressing CD83, indicating a larger proportion of CD40L-treated MoDC had matured (38-40% CD83-positive) compared to untreated MoDC populations (12-22% CD83-positive). Frequencies of MoDC expressing CD86, CD40, HLA DP DQ DR were high, >80%, and similar with or without CD40L treatment. In cells from one donor, there was marked increase in CD83, HLA class II, and CD86 expression/cell, by 75%, 30%, and 50% respectively, in CD40L-DC compared to untreated MoDC. For the other donor, the MFIs of these surface markers were similar in CD40L-MoDC and untreated MoDC.

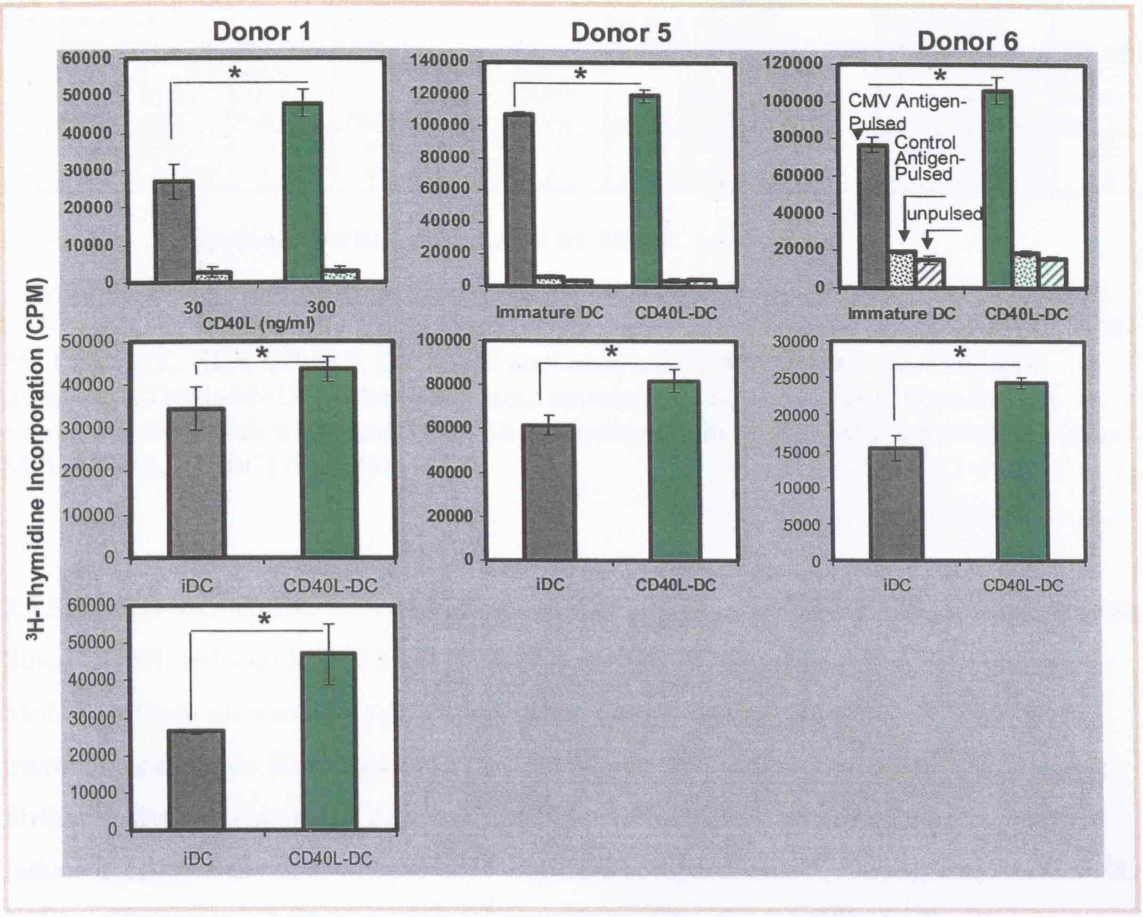

Figure 3.10 Induction of T Cell Proliferation by CD40L-Treated- Compared to Untreated MoDC.

Adherent Mo were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 for 7 days. On day 4, the differentiating MoDC were pulsed with antigen and treated with CD40L (300ng/ml) 3h later, or left untreated. On day 7, the cytokines were washed off and the MoDC were co-cultured with autologous PBL at a ratio of 1 : 10-20 in triplicate wells of 96-well plates for 6 days, in the presence of 1mCi ^{3}H-Thymidine/well for the final 16h before harvesting. ^{3}H-Thymidine incorporation was measured by scintillation counting. Results are displayed as average CPM of triplicate wells. **Error bars**, SD; **asterisks**, significant difference; **green**, CD40L-MoDC; **grey**, untreated MoDC; **solid bars**, DC pulsed with CMV antigen; **dotted bars**, DC pulsed with control antigen; **diagonal lines**, DC pulsed with no antigen.

Donor	CD83			CD40			CD86			HLA II		
	Expression Frequency (%)		MFI ratio	Expression Frequency (%)		MFI ratio	Expression Frequency (%)		MFI ratio	Expression Frequency (%)		MFI ratio
	MoDC	MoDC-CD40L	MoDC-CD40L/ MoDC	MoDC	MoDC-CD40L	MoDC-CD40L/ MoDC	MoDC	MoDC-CD40L	MoDC-CD40L/ MoDC	MoDC	MoDC-CD40L	MoDC-CD40L/ MoDC
1	22	42	1.2	91	99	1	83	92	1.1	93	92	N/D
3	12	38	1.75	90	83	1.3	83	85	2	98	98	1.3

Table 3.3 Surface marker expression by MoDC +/- CD40L.

300ng/ml CD40L were added to MoDC on day 4 of their 7-day differentiation with GM-CSF and IL-4. On day 7, DC were double- or single stained with anti-CD83-FITC, CD40-PE, CD86-PE, HLA DP, DQ, DR-FITC and analysed by flow cytometry. The table compares CD40L-MoDC to their untreated counterparts according to surface marker expression frequencies (%) and MFI. To normalise the data, The MFI is shown as a ratio: MFI (CD40L-MoDC) /MFI (MoDC).

3.3.5.5 CD40L-MoDC Induced Proliferation in CD4 and CD8 T Cell Compartments

Since CD40L induced higher levels of CMV-specific T cell proliferation than untreated MoDC, without increasing proliferation in response to control antigen, CD40L was a promising candidate for inclusion in the CMV-specific T cell culture system, warranting further analysis. Untreated CMV-antigen-pulsed MoDC have previously been shown to induce T cell proliferation in both CD4 and CD8 compartments[56]. To test whether CD40L had an effect on the dynamics of CMV-specific proliferation in different T cell compartments, post-culture on day 11, T cells from donor 8, induced by DC treated with CD40L or untreated, were stained for CD3, CD4, and CD8 markers and analysed by flow cytometry. As shown in figure 3.11, induction with CD40L-treated DC resulted in only a small change in the ratio of CD4:8 in the CD3-positive compartment compared to untreated MoDC, with a slightly increased frequency of CD8-expressing T cells. Six times as many T cells expressed CD4 than CD8 in the CD40L culture, which compared to a CD4:8 ratio of 7 in the culture using untreated MoDC.

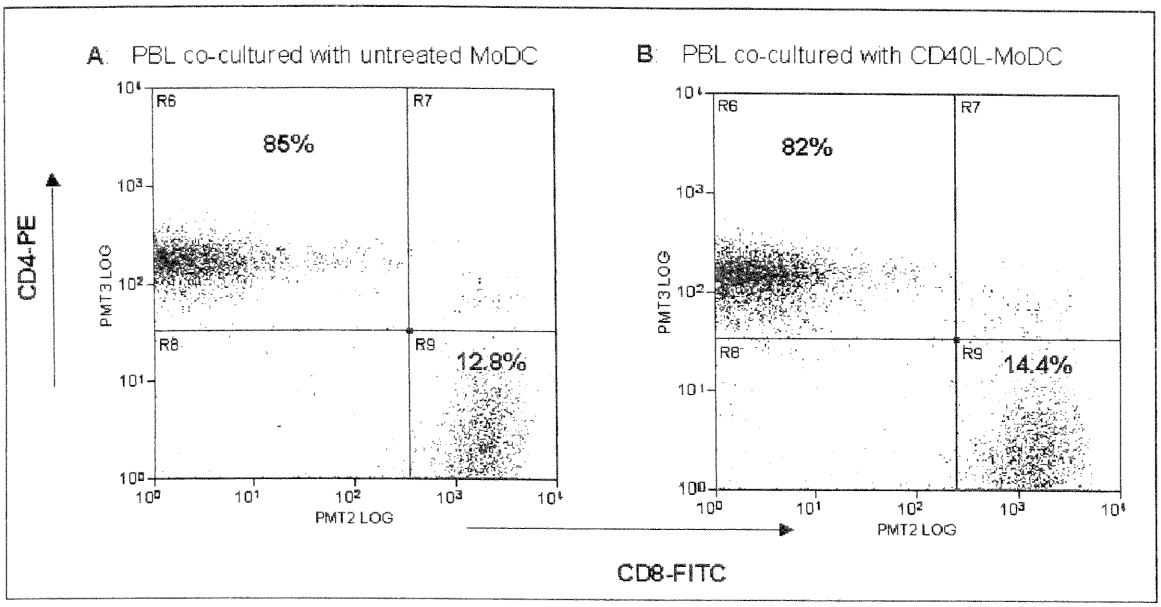

Figure 3.11 CD4/CD8 Expression by T Cells after Co-Culture with CMV Antigen-Pulsed MoDC, +/-CD40L.

Duplicate cultures of MoDC were pulsed with CMV antigen on day 4 of differentiation. One culture was treated with 300ng/ml CD40L, the other remained untreated. On day 7, washed MoDC were co-cultured with autologous PBL for 11 days, with one further restimulation with antigen-pulsed, untreated MoDC after 7 days. Post-culture PBL were stained with CD3-PE-Cy-5, CD4-PE, CD8-FITC mabs. Flow diagrams of 10000 events show the frequencies post-culture CD3+ T cells expressing CD4 or CD8. **A,** co-cultures with untreated MoDC; **B,** co-cultures with CD40L-treated MoDC.

3.3.6 LIGHT

3.3.6.1 LIGHT-MoDC were Potent Inducers of Autologous T Cell proliferation in Response to Antigen

CMV antigen-pulsed MoDC treated with a range of concentrations of LIGHT induced significantly greater T cell proliferation than untreated CMV antigen-pulsed MoDC from two different donors at the optimal concentration of 300ng/ml (Figures 3.12, 3.14).

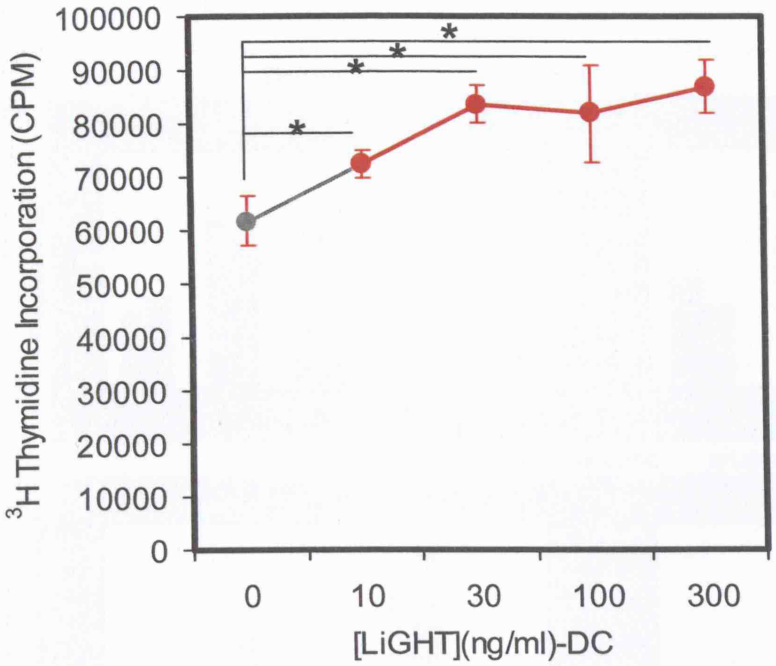

Figure 3.12 Antigen presentation/ T cell proliferation assay: Optimisation of Induction of T cell proliferation by LIGHT-treated MoDC.

Adherent Mo from CMV seropositive donor 5 were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 for 7 days. Differentiating MoDC were pulsed with CMV antigen day 4, and exposed to LIGHT at various concentrations for the last 48h, from day 5 onwards. On day 7, cytokines were washed off and MoDC were co-cultured with autologous PBL at a 1:10 ratio, in triplicate wells of a 96-well plate for 4 days in the presence of 1μCi of ^{3}H-Thymidine/well for the last 16h before harvesting. ^{3}H-Thymidine incorporation was measured by scintillation counting. Results are displayed as CPM (mean of triplicate wells). **Red**, LIGHT-DC; **grey**, untreated DC; **error bars**, standard deviation of means. **Asterisks**, significantly different from untreated control.

3.3.6.2 LIGHT-MoDC Did Not Differ from Untreated MoDC by Phenotype

Immunophenotyping MoDC from two donors revealed that frequencies of cells expressing CD83 were similar with or without LIGHT-treatment, ranging from 30% to 40% (Figure 3.13A). Frequencies of CD40-expression (>/=70%), CD86 expression (>70%) and HLA DP DQ DR (>80) expression were high, and broadly similar in LIGHT-MoDC and untreated MoDC (Figure 3.13A). Furthermore, there was no consistent difference in the amount of surface marker expressed per cell between untreated and LIGHT-treated MoDC in these two donors, as indicated by the MFI (Figure 3.13B).

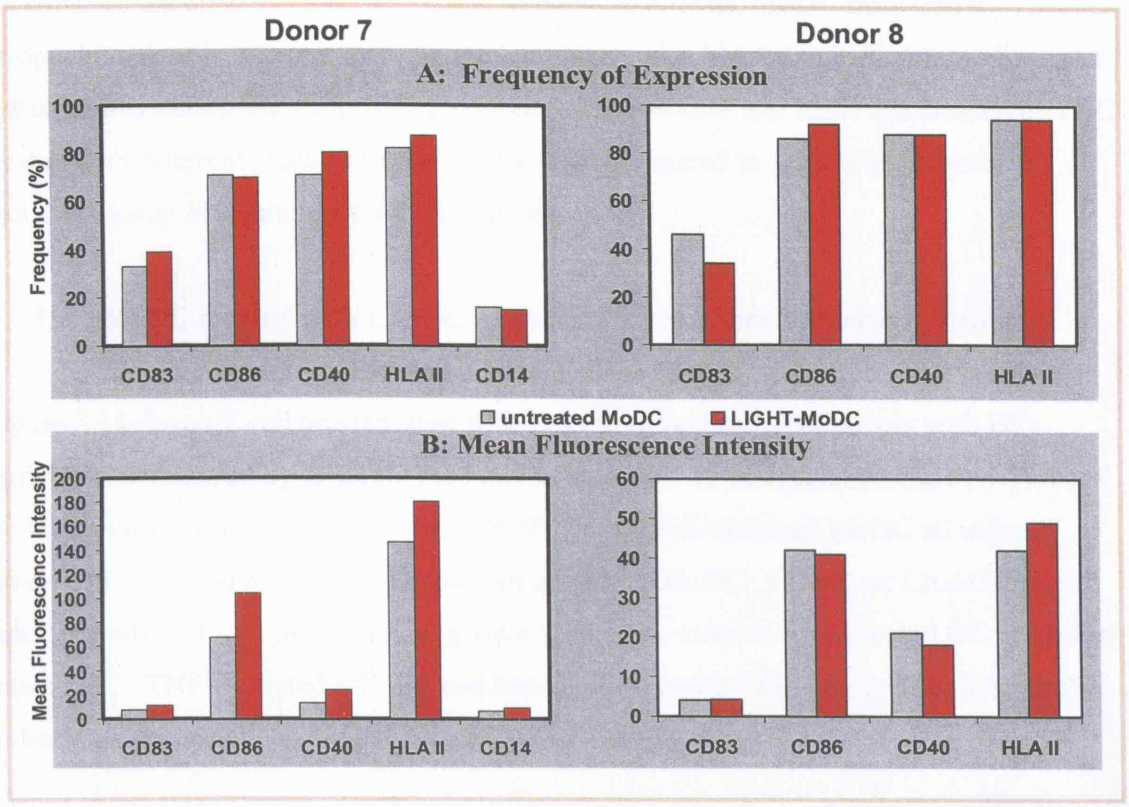

Figure 3.13 DC Immunophenotype: LIGHT-Treated MoDC.

Adherent Monocytes from donor 7, and Monocytes isolated by CD14-magnetic selection from donor 8 were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 for 7 days. On day 4, differentiating MoDC were treated with LIGHT (300ng/ml), or left untreated. On day 7, MoDC were stained with fluorescence-conjugated mabs against surface markers, and analysed by flow cytometry. **A**, Frequency of surface marker positive cells; **B**, mean fluorescence intensity. **Grey bars**, untreated MoDC; **red bars** LIGHT-MoDC.

3.3.7 Comparison of Different Maturation Agents

To compare the effects of various DC-maturation treatments, MoDC from CMV seropositive donors were set up in parallel cultures and subjected to different mediators or left untreated during the 7-day differentiation with GM-CSF and IL-4. Performance of DC treated with different maturation factors was then compared in a T cell proliferation induction assay with autologous T cells as responders.

3.3.7.1 MoDC treated with CD40L or LIGHT were better Inducers of Antigen-Specific T Cell Proliferation than TNF-α-MoDC

Figure 3.14 shows T cell proliferation with cells from two different donors with DC, immature, or matured by different methods as inducers. In both donors (Figure 3.14A and B), CMV antigen-pulsed and CD40L, LIGHT, or TNF-α–matured MoDC all induced levels of T cell proliferation above those of untreated MoDC. CD40L or LIGHT induced similar levels of T cell proliferation, greater than those induced by untreated DC or TNF-α-treated DC. TNF-α-treated DC induced intermediate levels of T cell proliferation, pitched in-between untreated DC and DC treated with CD40L or LIGHT.

3.3.7.2 There were No Additive or Synergistic Effects

Combining any two of these agents i.e. CD40L and LIGHT, CD40L and TNF-α, or LIGHT and TNF-α did not result in a further rise in T cell proliferation when compared to applying each agent alone, i.e. there was no additive or synergistic effect of combining maturation agents (Figure 3.14B).

Likewise, as shown in figure 3.15A, B, and C, combining CD40L, LIGHT, or TNF-α with PGE_2 for DC maturation resulted in no change in T cell stimulatory capacity compared to applying any one of these agents alone. Only when combining TNF-α with PGE_2, IL-1, and IL-6, a further increase in induction of T cell proliferation occurred (Figure 3.15C).

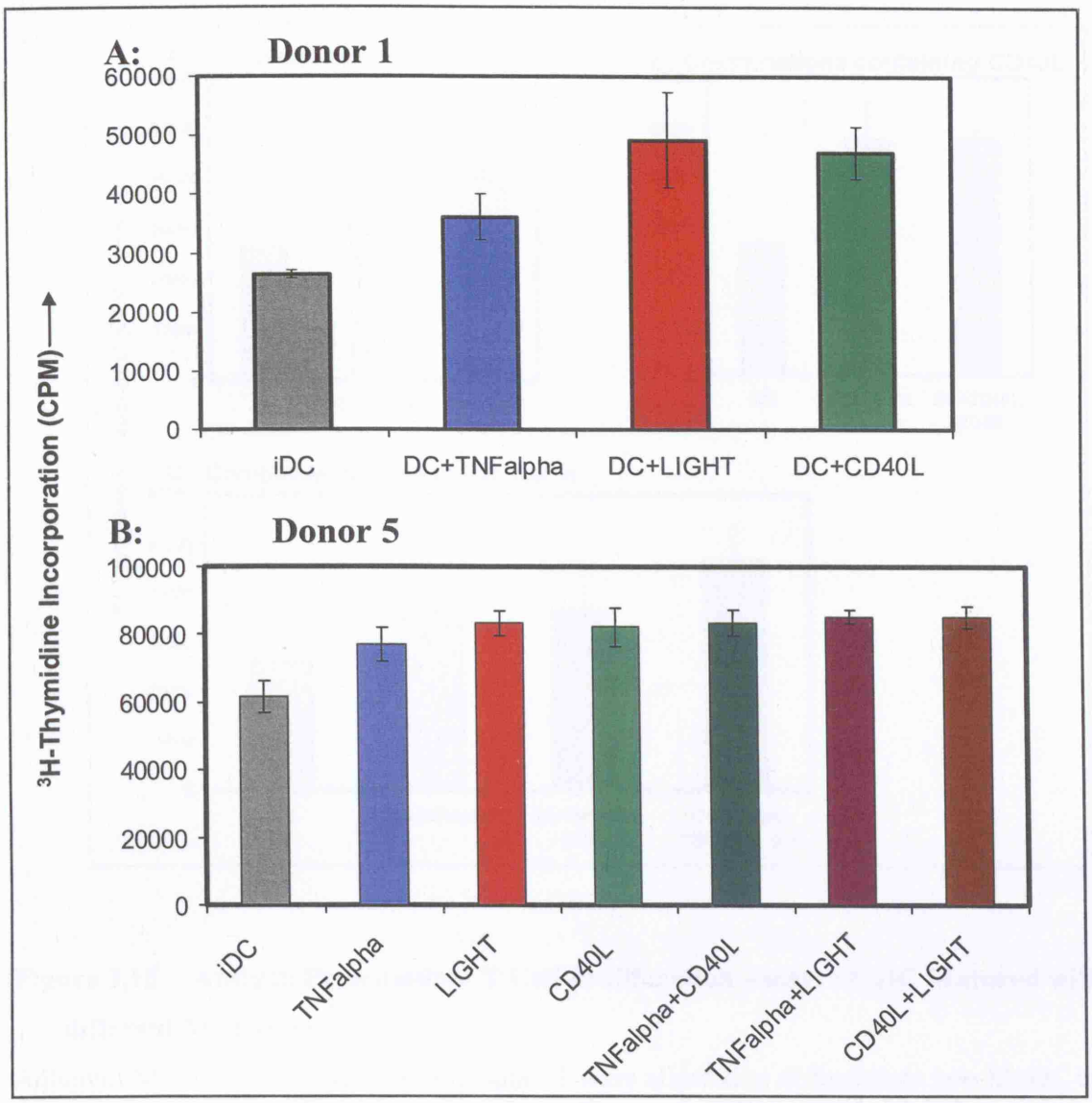

Figure 3.14 Antigen presentation/ T Cell Proliferation Assay: MoDC Matured with Different Mediators.

Adherent Mo were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 for 7 days. On day 4, the differentiating MoDC were pulsed with CMV antigen and TNF-α (100ng/ml), CD40L (300ng/ml), or LIGHT (300ng/ml) were added alone or in combinations after 3h. On day 7, the MoDC were washed and co-cultured with autologous PBL at a ratio of 1:10 or 20 in triplicate wells of 96-well plates. After 3 or 4 days of co-culture, 1μCi of ^{3}H-Thymidine/well was added before harvesting the plates on the next day. ^{3}H-Thymidine incorporation was quantified by scintillation counting. **A**, donor 1 (harvested day 5); **B**, donor 5 (harvested day 4).

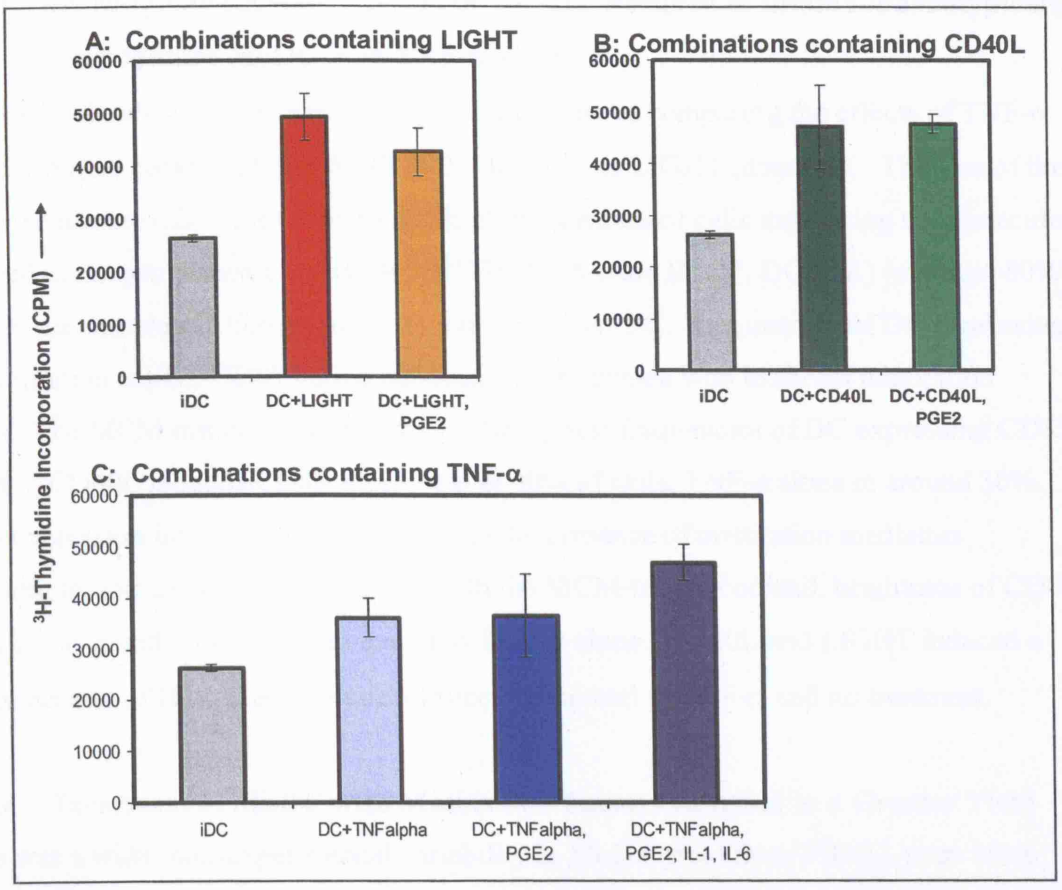

Figure 3.15 Antigen Presentation/ T Cell Proliferation Assay: MoDC Matured with different Mediators

Adherent Mo from CMV seropositive donor 1 were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 for 7 days. On day 4, differentiating MoDC were pulsed with CMV antigen. TNF-α (100ng/ml), CD40L (300ng/ml), or LIGHT (300ng/ml) were added 3h later. PGE_2 (1mg/ml), IL-1 (10ng/ml), IL-6 (10ng/ml) were added on day 6. On day 7, the MoDC were washed and co-cultured with autologous PBL at a ratio of 1:10 in triplicate wells of a 96-well plate for 5 days, in the presence of 1mCi of ^{3}H-Thymidine/well for the final 16h before harvest. ^{3}H-Thymidine incorporation was quantified by scintillation counting. **A**, MoDC pulsed with LIGHT/Light+PGE_2; **B**, MoDC pulsed with CD40L/CD40L+PGE_2; **C**, MoDC pulsed with TNF-α/TNF-α+PGE_2/MCM-mimic cocktail.

3.3.7.3 MCM-mimic cocktail, CD40L, or LIGHT Resulted in Immunophenotypically More Mature MoDC than TNF-α alone

Figure 3.16 shows surface marker expression frequencies comparing the effects of TNF-α to MCM-mimic cocktail (donor 6), CD40L (donor 7) or LIGHT (donor 3). The loss of the monocyte marker CD14 (not shown) and high frequencies of cells expressing the molecules involved in antigen presentation (CD40, CD86, HLA class II (DP, DQ, DR) (median>80%) indicate the complete differentiation of monocytes into DC. Frequencies of DC expressing the maturation marker CD83 varied between MoDC treated with different maturation agents. The MCM-mimic cocktail induced the highest frequencies of DC expressing CD83 (>70%). CD40L induced CD83 expression in 40% of cells, TNF-α alone in around 30%. CD83 expression frequency was increased in the presence of maturation mediators compared to untreated DC in all cases. With the MCM-mimic cocktail, brightness of CD86 staining was greatly increased compared to TNF-α alone. CD40L and LIGHT induced a greater density of HLA class II molecules/cell compared to TNF-α and no treatment.

3.3.7.4 Treatment of MoDC with Maturation Factors Resulted in a Greater Yield

There was a wide inter-experimental variability in MoDC yield from PBMC, even when cells were derived from the same donor on separate occasions. The output of untreated DC, for example, ranged from 13000 to 280000 DC/ million PBMC (median 25000) in 11 experiments using cells from 8 different donors. This variability may be attributed to differences between donors and/or variations the culture conditions, e.g. seeding density, or variability of media. Hence only comparisons within individual batches of MoDC generated at the same time under the same conditions, with the only variable being the maturation factors, were meaningful. Figure 3.17 shows normalised yield of MoDC treated with maturation factors compared to untreated MoDC (=baseline set as 100%). Exposure to maturation factors tended to increase the yield of MoDC when compared to untreated MoDC. This effect was greatest with the MCM-mimic cocktail and least with CD40L, where in 2/6 experiments yield was slightly less, whereas in 5/7 experiments the yield was greater than with untreated MoDC.

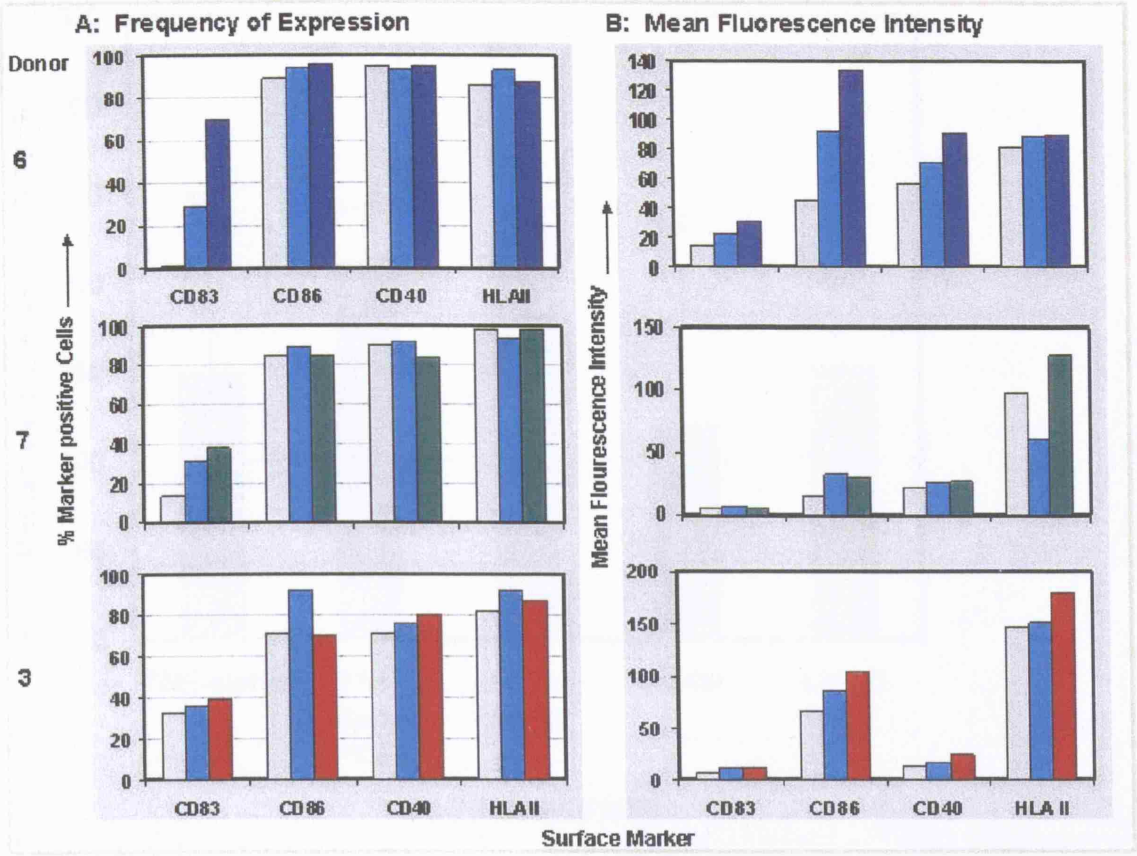

Figure 3.16 Comparison of Immunophenotypes of MoDC treated with various mediators of maturation.

Mo from 3 different donors were differentiated into MoDC for 7 days with GM-CSF and IL-4 and matured by various mediators in parallel cultures for each donor. **Grey**, untreated; **light blue**, treated with TNF-α (donors 6 and 7: 100ng/ml from day 4 donor 3: 20ng/ml from day 5); **dark blue**, MCM-mimic cocktail (TNF-α (100ng/ml from day 4), IL-1 (10ng/ml from day 6), IL-6 (10ng/ml from day 6, PGE_2 (1mg/ml from day 6)); **green**, CD40L (300ng/ml from day 6); **red**, LIGHT (300ng/ml from day 4). On day 7, MoDC were stained with anti- CD83, CD86, CD40, and HLA DP, DQ, DR, or isotype control mabs conjugated to FITC or PE. **A**, frequency of surface-marker positive cells; **B**, MFI of surface-marker positive cells.

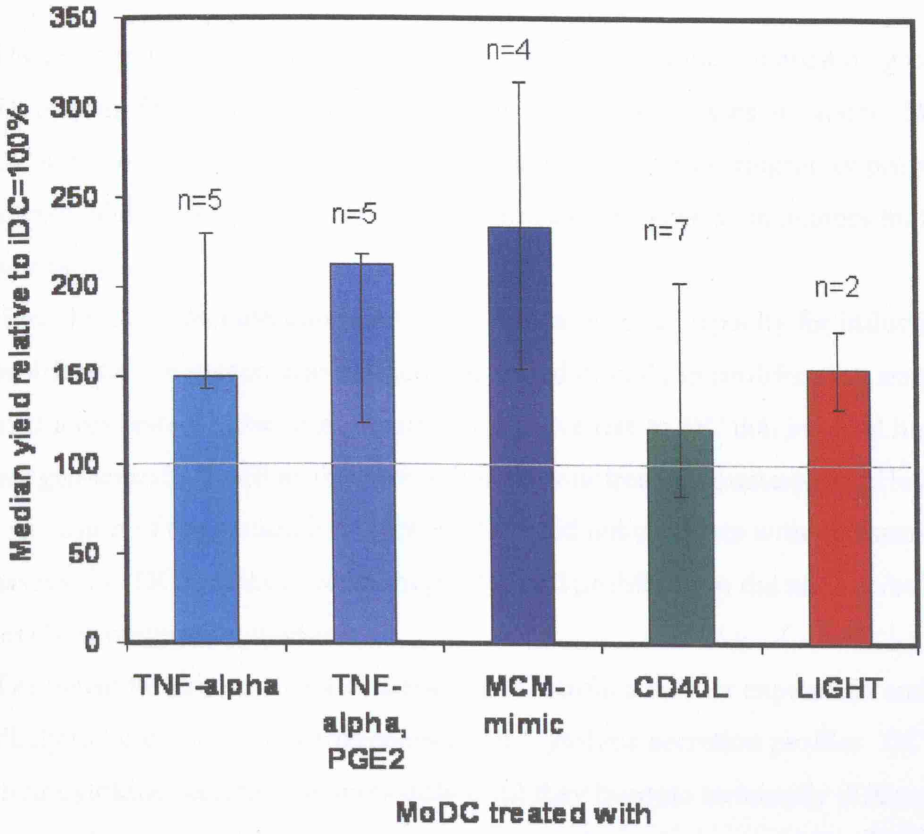

Figure 3.17 ***In Vitro*** **Generation of MoDC: Relative Yield.**

Median output of MoDC matured with various mediators relative to immature DC yield. MoDC treated with TNF-α, TNF-α and PGE_2, MCM-mimic cocktail, CD40L, or LIGHT were counted and compared to equivalent untreated MoDC generated in parallel from the same blood-draw. Data is expressed as % of untreated MoDC (**horizontal line**). **Error bars**, range.

3.4 Discussion

The *In vitro* induction of DC maturation has received much interest by groups involved in developing DC-based immunotherapy for infectious diseases or cancer. Measurements of DC surface marker expression, cytokine secretion profiles, migratory properties, antigen uptake, and induction of T cell proliferation can all serve as indicators for DC maturation (see table 3.1).

Here, for the direct measurement of DC function, their capacity for induction of T cell proliferation in response to antigen was tested directly in proliferation assays. Several of the mediators tested, alone or in combination, gave rise to DC that induced higher levels of antigen-specific T cell proliferation than their untreated counterparts. However, levels of expression of the maturation marker CD83 did not correlate with performance in functional assays; i.e. DC that induced the highest T cell proliferation did not express the highest levels of maturation markers.

The cause for this discrepancy between DC surface marker expression and function was likely to be the result of differences in DC cytokine secretion profiles. DC can modulate their cytokine secretion patterns only until they become terminally differentiated. Hence terminally mature DC can diverge in their cytokine secretion profiles[101;103], and are then resistant to further modulation[102]. Cytokine secretion profiles determine other functional properties of DC, and can ultimately greatly affect immunological outcome.

Between the immature- and terminally differentiated states, DC can be activated to varying degrees. *In vivo*, activated DC with immune-regulatory function, may produce high levels of cytokines, but remain *in situ*. These are also referred to as inflammatory-type DC. To achieve terminal differentiation DC need to migrate from tissue sites to lymph nodes (migratory-type DC). Partially matured DC can revert to less differentiated stages upon withdrawal of maturation factors. Even CD83 expression may be lost in some circumstances[101], which would suggest CD83 as a marker of DC differentiation, but not for terminal maturation.

Certain steps required *in vivo* to turn resting DC into highly efficient APC can be overcome in culture. This can be achieved by manipulating DC maturation using various mediators, in order to obtain MoDC with the desired effect on antigen-specific T cells.

The objective of this work was to identify mediators of DC activation or maturation that increase efficiency of CMV antigen-driven T cell cultures. This involves antigen presentation to memory T cells to induce their activation and proliferation, whilst maintaining specificity.
The naturally occurring barriers to *in vivo* DC-induced T cell activation serve as a safeguard against inappropriate T cell activation in response to self- or irrelevant antigens. Likewise, in culture systems for the propagation of pathogen-specific T cells it is important to ensure antigen-specificity. MLRs are widely used to test DC function, but they fail to address antigen-specificity. Measuring T cell proliferation in response to no antigen- or control antigen-pulsed DC showed that some *in vitro* matured DC induced higher levels of unspecific T cell proliferation than their less differentiated counterparts.

The characteristics of MoDC matured with the various mediators tested here are summarised in table 3.4. TNF-α, a naturally occurring proinflammatory mediator, has been used alone[79;101;103;106;108] or in combination with other factors for the induction of DC maturation. MoDC treated with TNF-α alone have previously been shown to become activated; they expressed CD83, showed superior performance to iDC in MLRs, and presented neo-antigen to autologous T cells, whilst having lost their capacity for FITC-dextran uptake [79]. However, evidence for only partial DC maturation induced by TNF-alpha was also presented[101]. TNF-α-matured DC could revert to a less mature state upon TNF-α withdrawal. CD83 expression ceased and there was a decline in the performance in functional assays.

Comparisons between findings from the many published studies are complicated by the variations in timing of exposure, lengths of exposure to TNF-α, and concentration of TNF-α, which can all influence the properties of the resulting DC. Schlienger et *al.*[79] showed that for maximum effect TNF-α had to be present in cultures of differentiating MoDC early on (from day 4) for a sufficiently long period of time (4 days). In none of the other studies was TNF-α applied to these specifications, so that for example the incomplete maturation observed by Nelson *et al.* may have been caused by insufficient exposure to TNF-α (from day 10 for 48h).

For the induction of CMV-specific T cells, DC treated with TNF-α at optimal levels of 100ng/ml applied from day 4 onwards induced higher levels of T cell proliferation than untreated MoDC. However, TNF-α-DC also induced excess non-CMV-specific T cell proliferation in one experiment, making TNF-α unsuitable for clinical applications, because of the increased risk of non-specific T cell activation.

	Immunophenotype			Functional		
	Maturation marker (CD83) expression	Markers associated with antigen presentation (CD86, CD40)	HLA class II expression	Antigen presentation (Induction of T cell proliferation		Yield
					Specificity	
untreated DC	+	++	++	++	++	+
TNF-alpha-DC	++	++	++	+++	?	++
TNF-alpha +PGE_2-DC	+++	+++	+++	++	?	+++
TNF-alpha, IL-1, IL-6, PGE_2-DC	+++	+++	+++	+++	+	+++
LIGHT	++	+++	+++	++++	?	++
CD40L	++	+++	+++	++++	++	++

Table 3.4 Summary of MoDC-Maturation.

Adherent monocytes were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 for 7 days. During differentiation, they were first pulsed with CMV- or control antigen, and then treated with various maturation agents. Typically on day 7, they were washed, counted, irradiated, and used as stimulators of autologous T cell proliferation in antigen presentation assays. DC were also stained with fluorescent mabs against surface markers and analysed by flow cytometry.

DC exposed to suboptimal levels of TNF-α repeatedly induced lower levels of CMV-specific T cell proliferation than untreated DC. We noticed a down-regulation of HLA class II expression in response to exposure to TNF-α. This was concomitant with the high frequencies of CD83-expression that would normally indicate maturity. This reduction in the numbers of HLA class II molecules available for antigen presentation to TCRs, may have been responsible for the inconsistent and low levels of T cell proliferation.

TNF-α and PGE_2 were evaluated alone[103] and as ingredients in an MCM-mimic cocktail with other pro-inflammatory cytokines. Treatment with just TNF-α and PGE_2 generated DC with very mature-type morphology and phenotype, with respect to CD83, CD40 and CD86 expression, compared to both untreated DC and DC treated with TNF-α alone. However, DC treated with this combination showed functional impairment, inducing levels of CMV-specific T cell proliferation equal to, or even below those induced by untreated DC. Since surface marker expression of MoDC treated with TNF-α and PGE_2 was indicative of mature DC, the observed inhibitory effect on antigen-specific T cell proliferation may be a consequence of the cytokine secretion profile induced by exposure to these two factors.

The cytokines secreted by activated MoDC contribute greatly to their functional properties further down the line. Many cytokines have autocrine effects, as well as effects on the T cells found in direct contact via TCR-MHC. These in turn produce cytokines affecting DC maturation. For example, DC producing predominantly IL-12 have been shown to induce IFN-γ-secretion in the T cells in contact, biasing the antigen-specific T cell response towards TH-1[123]. This effect of IL-12 is augmented by IL-4 [115]. In addition, evidence for IL-12-independent induction of TH-1 responses does also exist[124]. DC that secrete predominantly IL-10, on the other hand, are inhibitory to their own further maturation and to TH-1 responses[125].

The combination of factors PGE_2 and TNF-α in the absence of other cytokines gives rise to functionally impaired MoDC, whereas as part of the MCM-mimic cocktail these two factors are associated with powerful induction of T cell proliferation. PGE_2, a phospholipid breakdown product released at sites of tissue injury, is a modulator of DC function. In combination with other mediators, but not alone, PGE_2 has been shown to induce differentiation of immature DC into migratory-type DC which were fully mature, and hence resistant to further modulation of their cytokine secretion patterns[103;112;113]. Depending to a large extent on the sequence of pro-inflammatory mediators they have encountered during activation, these migratory-type DC have been shown to often secrete lower levels of cytokines than non-migratory, inflammatory-type DC, and it has been suggested that they were likely inducers of type-2 responses[112]. However, high-level IL-12 production

associated with PGE_2 has also been reported[110]. Our results seem to confirm that PGE_2 applied to MoDC without sufficient co-stimulation through other pro-inflammatory mediators can have an inhibitory effect on the induction of T cell activation.

PGE_2 is a regulator of DC migration to lymph nodes[103]. It is possible that the migratory step itself, which cannot take place in this *in vitro* system, contributes to final DC maturation. *In vivo*, DC producing only low levels of cytokines may still be capable of activating antigen-specific T cells in the very specialised microenvironment of the lymph nodes.

Cultures are likely to impose a different set of demands, where migration usually does not play a part. Here, for optimal enhancement of memory T cell responses in culture, cytokines secreted by MoDC may be of greater value than migratory properties. Compared to migratory-type DC, the non-migratory/pro-inflammatory-type DC have been shown to secrete larger amounts of cytokines, induce γ-IFN-production in T cells, and are thought to bridge innate and adaptive arms of the immune response at sites of tissue injury or pathogen entry[103]. These pro-inflammatory-type DC may be more effective at inducing antigen-specific memory T cell responses in this culture setting.

Inclusion of IL-1 and IL-6 in this cocktail, on the other hand, induced DC maturation defined by both phenotype and function [80;97;104]. Our data also show that MoDC treated with this cocktail had a tendency to induce non-specific T cell proliferation, and could not be regarded as safe for use in immunotherapy.

LIGHT is a member of the TNF superfamily that, like CD154, is up-regulated on activated T cells. Using a CD154 and LIGHT- transduced cell line for induction of DC maturation, LIGHT has been shown to co-operate with CD40L in the induction of DC maturation, but to have only a limited effect by itself [109].

Here we show that as a soluble factor, LIGHT may have a similar effect on DC maturation as soluble CD40L, with no further improvement achieved with the combination of both soluble factors. However, the specificity of the T cell proliferation induced by LIGHT-MoDC remains to be tested.

The ligation of CD40 on the DC plasma membrane provides the first signal for DC maturation[111]. This signal is normally supplied by the interaction of CD154 on CD4+ T cells with CD40 on immature DC. Alternatively, soluble CD154 supplied in culture can provide a similar stimulus for DC maturation, in theory cutting out the necessity for CD4+ T cells.

In our hands, MoDC treated with CD40L were much more heterogeneous in their surface marker expression than MoDC treated with the TNF-α and PGE_2-containing cocktails, with only about 40% expressing CD83. Nevertheless, CD40L-treatment resulted in the most effective induction of autologous T cell proliferation by DC with the highest antigen-specificity, compared to DC treated with all other agents tested here.

Since CD40L-treated MoDC were less mature defined by surface marker expression than TNF-α/PGE_2-treated MoDC, the functional difference was likely to have resulted from different cytokine secretion profiles induced by the different mediators. Evidence for this was given by Ebner *et al.*[115]who reported that CD40 ligation was sufficient for induction of IL-12 production whereas MCM was not.

The requirement for cross-linkage of CD40 on the DC plasma membrane as one of the signals for induction of DC maturation suggests that the use of CD40L in its trimeric form is likely to induce even more potent and more homogeneous MoDC. CD40L was the best candidate for routine inclusion into the culture system for generation of CMV-specific T cell lines.

CD40L-mediated activation of MoDC has been reported to be enhanced by other factors, such as LIGHT[126 109], or IFN-α [116]. However, under the conditions employed here, combining CD40L with TNF-α, LIGHT, or PGE_2, resulted in no further improvement of DC performance in autologous antigen-presentation assays.

Induction of T cell proliferation serves as a functional test for DC, but conveys no information about the properties of the proliferating T cells. Changing the type of APC could potentially lead to a change in type and function of responding T cells, so their further characterisation was required.

In vitro expanded CMV-specific T cells would need to have antigen-specific cytotoxic properties, as well as to provide helper functions for the maintenance of immunity. We have previously shown that untreated MoDC induced proliferation in both CD4 and CD8 T cell compartments, and the resulting T cells demonstrated antigen-specific cytotoxicity[56]. This CMV-specific cytotoxicity has been shown to be HLA class 1 restricted, i.e. mediated by CD8+ T cells. The relative proliferation in CD4 and CD8 T cell compartments induced by CD40L-MoDC and untreated MoDC was shown here to be very similar, which would suggest sufficient induction of proliferation in both CD8 and CD4 T cell compartments. The characteristics of CMV antigen-specific T cell lines induced by CD40L-DC are explored in greater detail in Chapter 4.

In conclusion, out of the various mediators of DC maturation tested here, CD40L was the most promising candidate for enhancing induction of donor-derived CMV-specific T cell lines for adoptive cell therapy. CD40L-treated DC, although not fully mature, consistently induced high levels T cell proliferation in response to CMV-, but not control antigen. DC treated with the other mediators were either inconsistent inducers of T cell proliferation, inducers of non-CMV-specific proliferation, or both. The one possible exception was LIGHT, which requires further evaluation. However, the possibilities of altering relative timing and dosing in the multi-mediator combinations are infinite, and further optimisation of the conditions may well be possible.

CHAPTER 4

Modifications to the Established Protocol for the *In Vitro* Generation of CMV-Specific T Cell Lines

4.1 Introduction

The culture system we previously developed for the *in vitro* expansion of CMV-specific T cell lines uses peripheral blood from CMV seropositive donors as starting material [56]. Essentially, CMV antigen-pulsed monocyte-derived dendritic cells (MoDC) are co-cultured with autologous peripheral blood lymphocytes (PBL) for two to three weeks, with one re-stimulation with CMV antigen-pulsed DC per week, resulting in activation and proliferation of CMV-specific T cells. Post-culture cells can then be given intravenously to stem cell transplant recipients at a dose of 1 $x10^5$/kg body weight to aide the restoration or the introduction of immunity against CMV, whilst minimising the risk of graft versus host reactivity. A diagram of the original protocol is shown in figure 4.1.

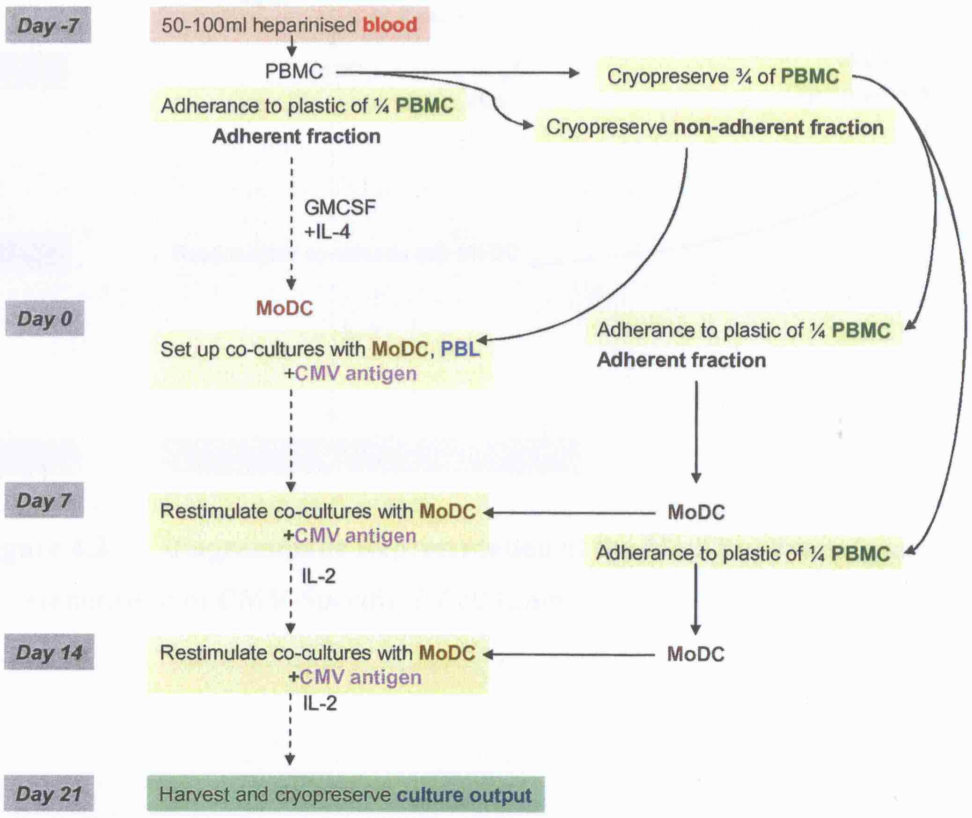

Figure 4.1 Diagrammatic Representation of the Original Procedure for the Generation of CMV-Specific T Cells.

Some details of the culture conditions have been altered since the beginning of the first clinical trial in an attempt to make the system more robust and reliable, allowing the generation of sufficient numbers of CMV-specific T cells in a minimum amount of time. A diagram of the current protocol is shown in figure 4.2. Experiments leading to these alterations are described below.

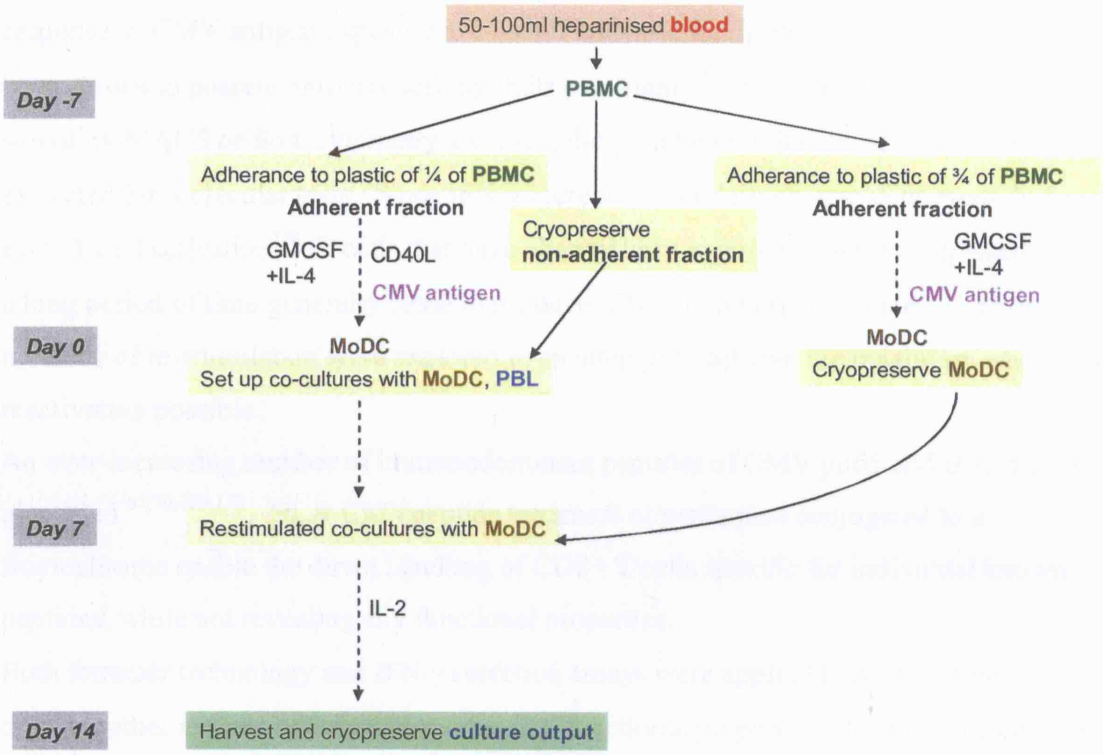

Figure 4.2 Diagrammatic Representation of the Modified Procedure for the Generation of CMV-Specific T Cell Lines.

Little is known about the properties of the T cells generated in this culture system. Apart from CMV-specific T cell proliferation, CMV-specific and HLA class I-restricted lysis of target cells by culture output cells has been demonstrated *in vitro.*

Cytotoxic T cell responses have been shown to be crucial in the control of CMV infection [127]. IFN-γ is secreted by CD4+ or CD8+ T cells committed to TH1-type immune responses, and labelling T cells for IFN-γ secretion can give an indication of the potential for induction of TH1-type responses. Hence, IFN-γ secretion by post culture cells in response to CMV antigen exposure is a useful indicator for T cell function, and IFN-γ has been shown to possess antiviral activity in its own right [72]. Because IFN-γ secreting T cells sorted by MACS or flow cytometry are alive, they can be re-cultured, or their nucleic acids extracted for molecular tests. Since IFN-γ secretion is a relatively transient event during early T cell activation[128], T cells that have already been exposed to antigen repeatedly over a long period of time generally cease to produce IFN-γ, unless re-stimulated. Various methods of re-stimulation were explored in an attempt to achieve the maximum amount of reactivation possible.

An ever-increasing number of immunodominant peptides of CMV pp65 and IE-1 are being identified [65;70;129;130]. HLA-CMVpeptide tetramers or pentamers conjugated to a fluorochrome enable the direct labelling of CD8+ T cells specific for individual known peptides, while not revealing any functional properties.

Both tetramer technology and IFN-γ secretion assays were applied to post-culture T cells to draw together aspects of their phenotypic and functional properties. T cells isolated from the culture output by both methods were characterised by comparative TCR CDR3 spectratyping. Post-culture IFN-γ secreting T cells were re-cultured to test their proliferative potential.

4.2 Results

4.2.1 CD40L

4.2.1.1 CD40L Matured MoDC were Capable of Inducing CMV Specific T Cell Proliferation even when Immature MoDC were Ineffective

As described in chapter 3, MoDC matured with CD40L were found to have superior capacity for inducing antigen-specific T cell proliferation than their counterparts not exposed to CD40L.

To test the effect of CD40L in longer-term cultures, cultures from one CMV-seropositive donor were set up in duplicate. Mo differentiating into DC were pulsed with CMV antigen on day 4. After 3 hours, 300ng/ml CD40L was added to one of the cultures. Co-cultures with autologous T cells were started after the 7-day differentiation of MoDC, and maintained for 14 days with one further re-stimulation with CMV antigen-pulsed DC. During this period, T cells proliferated in the CD40L-culture: cell counts rose 13-fold, from 2 to 27 million. At the same time, there was no growth in the culture without CD40L. CD40L-maturation did not markedly alter CD4/8 ratios of post-culture T cells (see Figure 3.11), or post-culture TCR CDR3 spectratype patterns (Figure 4.3), indicating proliferation of the same CMV-specific T cell clones with and without CD40L. When the culture protocol was changed to using CD40L-matured MoDC for the initial induction of CMV-specific T cell growth, the success rate of increased from 45% in the 20 cultures before introduction of the change, to 65% in 20 cultures following the change.

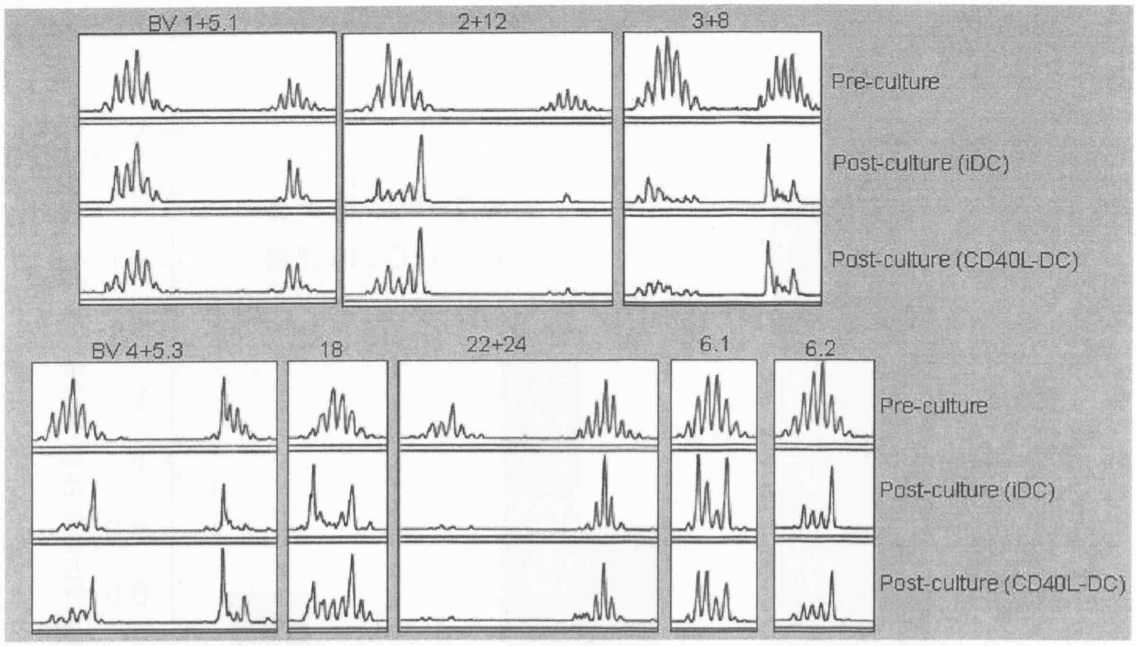

Figure 4.3 TCR CDR3 spectratypes of CMV-specific T cells induced by immature or CD40L-DC

T cells were co-cultured for 14 days with immature DC or DC treated with CD40L. cDNA from pre- and post-culture T cells was amplified by TCR CDR3 spectratyping PCR, using primers specific for 23 different BV families in combination with a BC-region primer. The panels show the size distribution of single or duplex PCR products for a total of 13 BV families.

4.2.1.2 CD40L-Matured MoDC Induced IFN-γ Secretion in a Higher Proportion of T Cells than iDC

T cells secreting IFN-γ give rise to TH1-type responses, such as those associated with control of CMV infection. Post-culture IFN-γ secretion by T cells was measured using the cytokine secretion assay (chapter 2, section 2.10).

T cells from Day 7 co-cultures with either CD40L-DC or untreated DC were assayed after a 16-h re-stimulation with antigen-pulsed CD40L-DC. As shown in figure 4.4, a greater proportion of T cells from CD40L-DC co-cultures secreted IFN-γ than from iDC co-cultures. The difference was greatest in the CD8 T cell compartment, with a nearly 50% increase in frequency of IFN-γ-secreting T cells. In the CD4+ T cell compartment there was

a greater than 20% increase in the frequency of IFN-γ-secreting T cells from CD40L-DC cultures compared to untreated DC co-cultures.

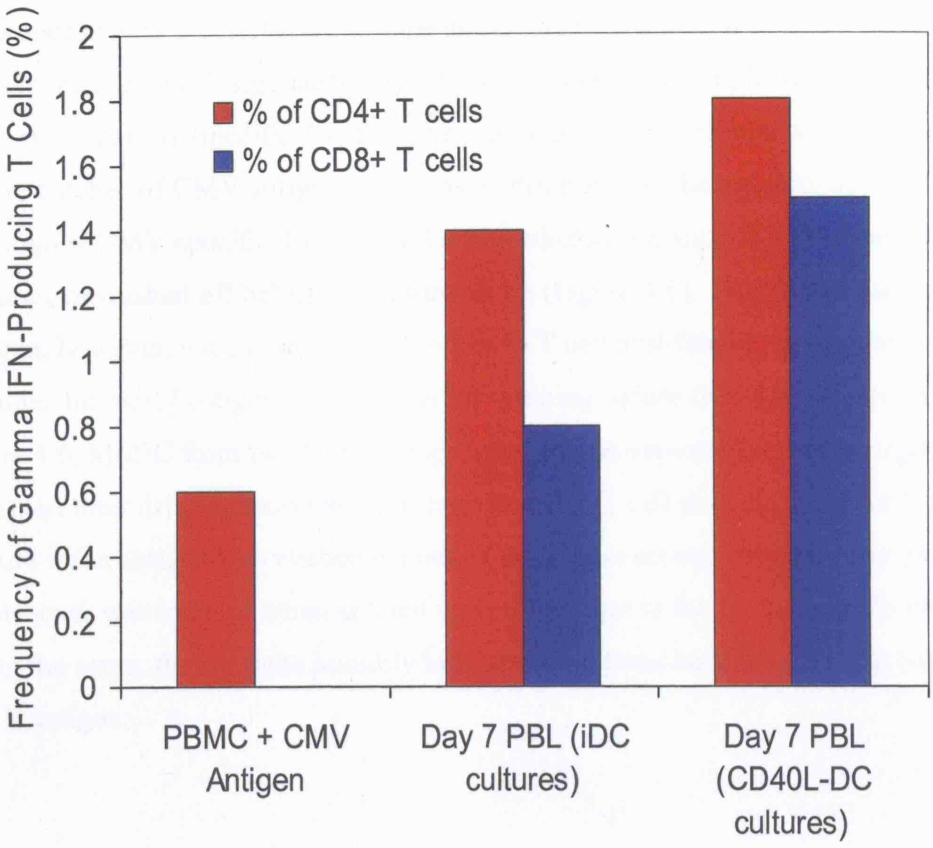

Figure 4.4 Levels of IFN-γ-Secreting T Cells Induced by Immature or CD40L-DC

T cells from CMV antigen-driven cultures (Donor 5), induced by either untreated- or CD40L-treated MoDC were characterised using the IFN-γ secretion assay. Thawed day-7 (donor 5) lymphocytes were restimulated with control antigen-pulsed or CMV antigen-pulsed CD40L-matured MoDC 16h before gamma-capture. IFN-γ-secretion in PBMC induced by control antigen was subtracted from all experimental values. Pre-culture levels of CMV-specific IFN-γ-secreting T cells were also determined in PBMC. The columns show frequencies of CMV antigen-induced IFN-γ-secreting T cells as a percentage of CD4 (**red**) or CD8 (**blue**) T cells.

4.2.2 Exposure to CMV Antigen: The Effect of Soluble CMV Antigen on T Cell Proliferation

Because immature DC take up antigen more effectively than mature DC, the introduction of a maturation step necessitated a change in the timing of addition of CMV antigen. To assess whether this change made any difference to DC function, MoDC were tested as inducers of antigen-specific T cells. The proliferation assay results were conflicting. With earlier batches of CMV antigen there was no difference in the capacity of MoDC to induce autologous CMV-specific T cell proliferation whether the antigen was present in the co-cultures, or washed off before co-culture set-up (Figure 4.5). Some later batches of CMV antigen, however, were extremely inhibitory to T cell proliferation when present in co-cultures, but not if antigen was removed by washing before co-culture setup. As shown in figure 4.6, MoDC from two CMV seropositive donors exposed to CMV antigen for the last 3 days of their differentiation induced high levels of T cell proliferation after 6 days of co-culture if the antigen was washed off before co-culture set-up. However, no proliferation whatsoever was induced when antigen was still present in the co-cultures. Everything else being the same, these results possibly indicated variations between different batches of CMV antigen.

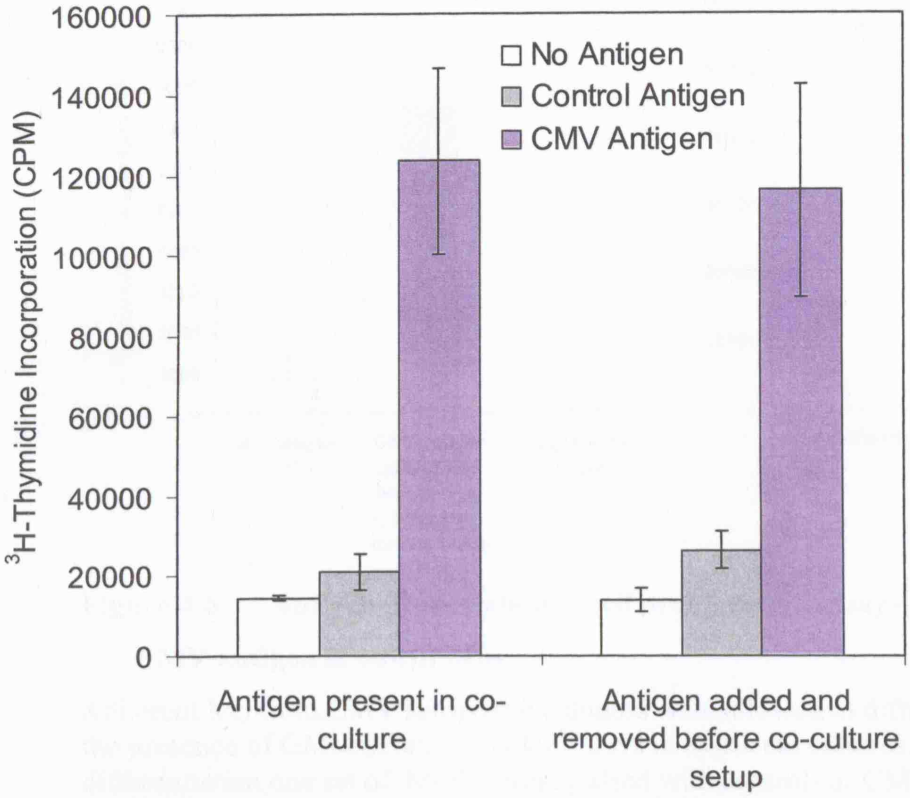

Figure 4.5 T cell proliferation/ antigen presentation assay: Effect of presence of CMV antigen in the co-cultures.

Adherent Mo (Donor 6) were differentiated in the presence of GM-CSF and IL-4 in a 6-well plate for 8 days. One set of wells was treated with CMV antigen or control antigen on day 6. After the 7-day differentiation, antigen-pulsed and un-pulsed MoDC were collected and co-cultured with autologous PBL in triplicate wells of 96-well plates (10000 DC/well, 200 000 DC/well). CMV lysate, control lysate, or no antigen were added to the co-cultures with DC not previously exposed to antigen. Co-cultures were maintained for 6 days, with ^{3}H-Thymidine (1μCi/well) present for the last 16h. Wells were harvested and ^{3}H-Thymidine incorporation quantified by scintillation counting. **Open bars**, DC pulsed with no antigen; **grey bars**, DC pulsed with control antigen; **purple bars**, DC pulsed with CMV antigen.

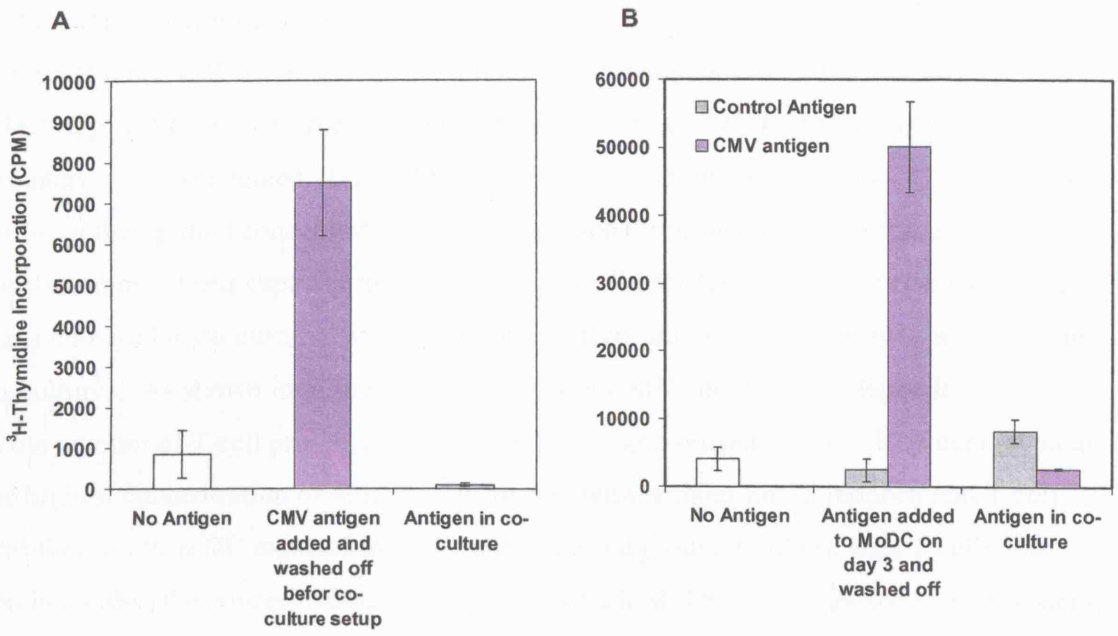

Figure 4.6 Antigen presentation/T cell proliferation assay – effect of presence of CMV antigen in co-cultures

Adherent Mo from CMV seropositive donors were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 for 7 days in duplicate cultures. On day 3 of differentiation one set of MoDC were pulsed with control- or CMV antigen, the rest was left un-pulsed. On day 7, MoDC were washed, irradiated, and co-cultured with autologous PBL in triplicate wells of 96-well plates. Control- or CMV antigen were added to co-cultures with DC not previously exposed to the antigens. **A,** donor 15: co-cultures of 1500 DC with 100 000 PBL maintained for 7 days; **B,** donor 12: co-cultures of 10000 DC/well and 100 000 PBL/well were maintained for 8 days. **A** and **B**, 1μCi ^{3}H-Thymidine/well was present for the last 16h of culture. Cultures were harvested and ^{3}H-Thymidine incorporation was quantified by scintillation counting. **Open bars**, DC pulsed with no antigen; **grey bars**, DC pulsed with control antigen; **purple bars**, DC pulsed with CMV antigen; **error bars**, means of triplicate wells.

CMV antigen present in co-cultures at concentrations above the optimal 1mg/ml total protein (Figure 4.7) was found to be inhibitory to T cell proliferation in two assays. The effect of pulsing DC with greater concentrations of antigen whilst reducing time of exposure was investigated. Day-7 MoDC from CMV seropositive donor 1 were exposed for 3h to the optimal concentration of CMV antigen (1mg/ml) to up to 4 times this concentration. Their capacity for induction of T cell proliferation under these conditions was compared to co-cultures with the normal concentration of CMV antigen added to the co-cultures. As shown in figure 4.8A, there was a CMV antigen-dose dependent increase in the amount of T cell proliferation induced by antigen-pulsed MoDC. However, even at the highest concentration of 4mg/ml, DC pulsed with antigen for 3h induced less T cell proliferation than DC exposed to CMV antigen throughout co-culture with T cells. In another assay, the concentrations of antigen to which MoDC were exposed to for the same length of time (3h) were taken to up to 10mg/ml (Figure 4.8B). At concentrations above 4mg/ml CMV antigen, the dose-dependent increase in T cell proliferation levelled off. DC pulsed with 10mg/ml CMV antigen induced similar levels of autologous T cell proliferation as DC in the standard setup with 1mg/ml CMV antigen added to co-cultures, and not significantly more than MoDC pulsed with 4mg/ml CMV antigen. Hence, exposure of MoDC to 1mg/ml CMV antigen for a longer period of time was more effective than exposure to higher concentrations of CMV antigen for 3h.

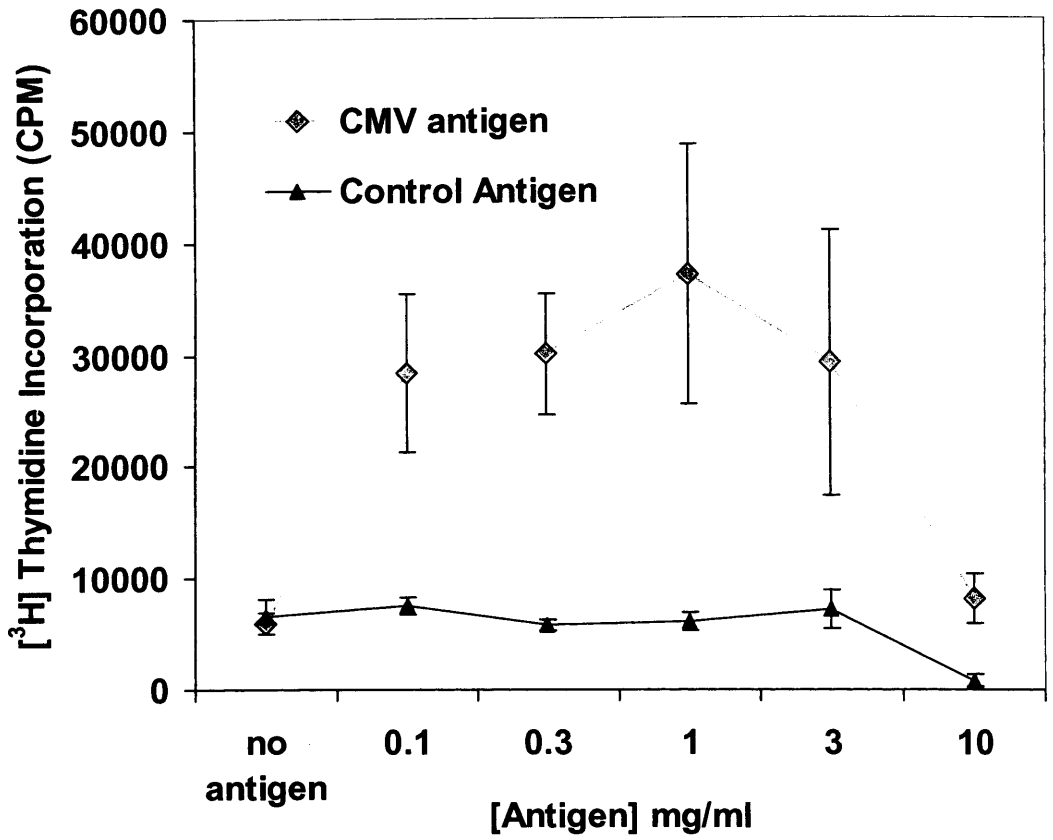

Figure 4.7 Antigen Presentation/ T Cell Proliferation Assay: Antigen Concentration Profile.

Mo from CMV seropositive donor 1 were allowed to differentiate into MoDC in the presence of GM-CSF and IL-4 for 7 days. Co-cultures of MoDC (10000) were set up with autologous PBL (200 000) in triplicate wells of 96-well plates. CMV antigen and control antigen were added to triplicate wells at a range of concentrations. The co-cultures were maintained for 6 days, with ^{3}H-Thymide present for the last 16h. Cultures were harvested onto a filtermat, and incorporation of ^{3}H-Thymidine into nucleic acids was quantified by scintillation counting.

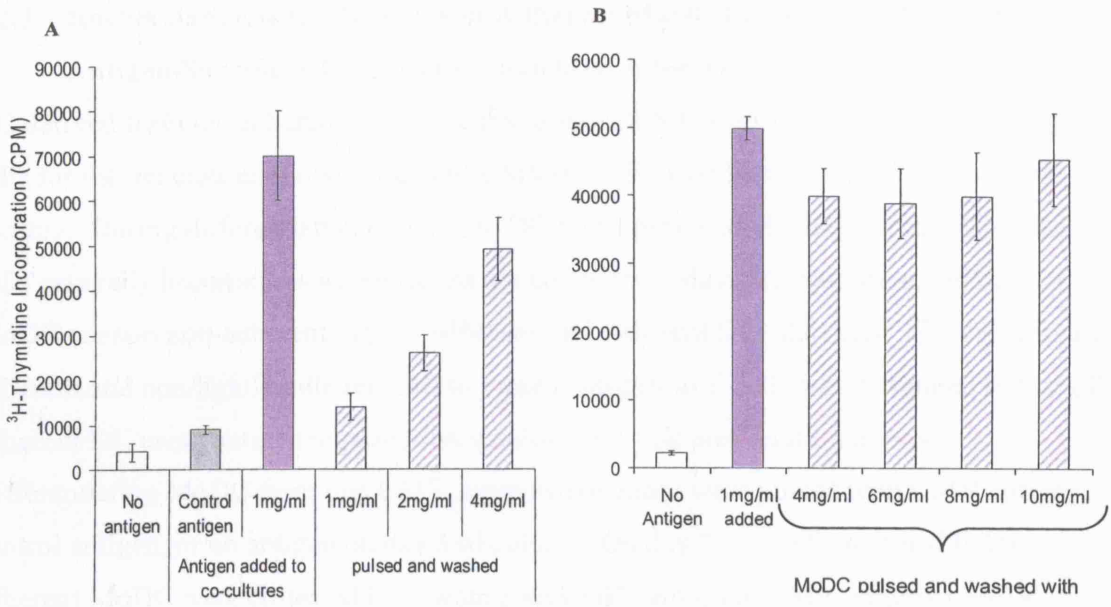

Figure 4.8 T Cell Proliferation/Antigen Presentation Assay: DC Pulsed and Washed with Different Concentrations of CMV Antigen

Adherent Mo from CMV seropositive donor 6 were differentiated in the presence of GM-CSF (100ng/ml) and IL-4 (100ng/ml) in 6-well plates. On day 7, MoDC were exposed to various concentrations of the CMV antigen for 3h, or left un-pulsed. MoDC were then collected and co-cultured with autologous PBL in triplicate wells of 96-well plates at 10000 DC/well with **A**, 100 000 PBL/well; **B**, 200000 PBL/well. CMV antigen (1mg/ml) or no antigen was added to wells of un-pulsed DC. Co-cultures were maintained for 6 days, with ^{3}H-Thymidine (1μCi/well) present for the last 16h. Wells were harvested and ^{3}H-Thymidine incorporation quantified by scintillation counting. **Solid bars**, antigen added to co-cultures; **striped bars**, CMV antigen washed off before co-culture; **open bars**, no antigen; **error bars**, SD of triplicate wells.

4.2.3 Restimulation with MoDC: Non-Adherent MoDC Induced Much Greater Antigen-Specific T Cell Proliferation than Adherent MoDC

DC derived from the adherent monocyte fraction of PBMC served as antigen presenting cells for the generation of donor-derived CMV-specific T cell lines for adoptive cellular therapy. During differentiation of Mo into DC in the presence of GM-CSF and IL-4, the cells generally become less adherent. At the end of the 7-day differentiation, cultures of MoDC contain non-adherent, lightly adherent, and adherent DC-like cells. The capacity of adherent and non/lightly-adherent DC to present antigen to T cells was compared to that of adherent DC using antigen presentation/autologous T cell proliferation assays. Differentiating MoDC from one CMV seropositive donor were pulsed with CMV antigen, control antigen, or no antigen on day 3 of culture. On day 7, non-adherent and lightly adherent MoDC were collected by washing with PBS w/o Ca and Mg, adherent MoDC were collected separately into PBS w/o Ca/Mg using a cell scraper. Viability of both populations was checked by Trypan blue exclusion. The ^{3}H-Thymidine incorporation assay was set up essentially as described in General Methods chapter 2. Co-cultures were maintained for 5 days, with ^{3}H-Thymidine present for the final 16h. As shown in figure 4.9, the adherent fraction induced no significant CMV-specific T cell proliferation above the control background, whereas non- or semi-adherent DC induced CMV-specific T cell proliferation about 5-fold above the control antigen background at this time point. Consequently, the non-adherent fraction of day-7 DC was routinely used for the re-stimulation of donor co-cultures.

4.2.4 Efficiency of *in vitro* DC Generation: Cryopreserved MoDC Generated from Fresh PBMC Give a Greater Yield than MoDC Generated from Thawed PBMC

Co-cultures for the generation of CMV-specific T cell lines are set up with MoDC derived from freshly isolated PBMC. A second batch of antigen-pulsed DC is required for restimulation of the T cells on day 7 of co-culture. Since acquisition of another fresh blood sample from the donor at the right time would be impracticable, a cryopreservation step is required. Mo from fresh PBMC can be differentiated into DC, pulsed with CMV antigen and cryopreserved until use. Alternatively, PBMC can be cryopreserved, thawed and

differentiated into DC. The yield of cryopreserved-thawed CMV antigen-pulsed MoDC was more than 3-fold greater than that of DC generated from cryopreserved PBMC from the same blood draw from the same donor. Total T cell counts in day 13 co-cultures were equal in cultures with both types of MoDC, having risen 3-fold to 30 million.

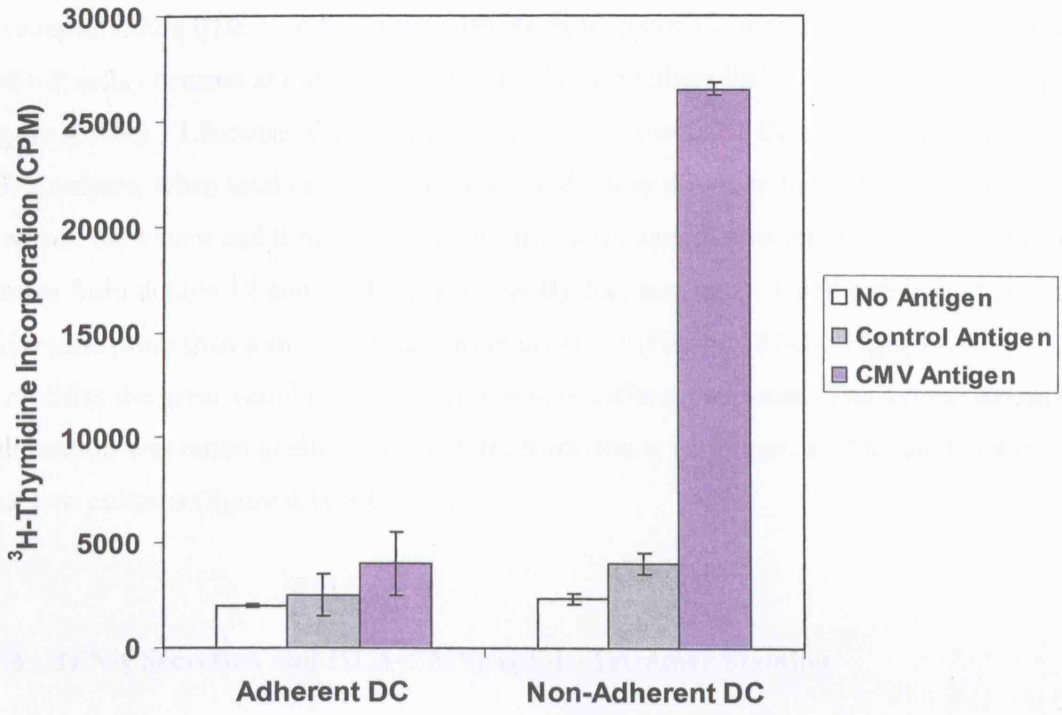

Figure 4.9 Antigen Presentation by Adherent or Non-Adherent MoDC.

Adherent Mo from CMV seropositive donor 10 were differentiated into MoDC in the presence of GM-CSF and IL-4 for 7 days. On day 4, differentiating MoDC were pulsed with CMV or Control antigen, or left unpulsed. On day 7, non-adherent and adherent MoDC were collected separately, washed and irradiated. 3000 MoDC were co-cultured with 200 000 autologous PBL in triplicate wells of 96-well plates. Co-cultures were maintained for 5 days, in the presence of 1μCi ^{3}H-Thymidine/well for the last 16h. Cultures were harvested onto filter paper, and ^{3}H-Thymidine incorporation into nucleic acids was quantified by scintillation counting.

4.2.5 Dynamics of T Cell Proliferation and Death in CMV Antigen-Driven Cultures

Our CMV antigen-driven T cell cultures are typically maintained for two weeks, with one restimulation on day 7. Figure 4.10 shows the development of cell numbers after restimulation during the second week of culture in three donors. As seen in all 3 donor cultures, T cells underwent a phase of proliferation, followed by a slow down in growth, and finally a reduction in cell number. In all donor cultures shown, CD4 T cells divided more rapidly than CD8 T cells. In the cultures from donor 12, maximum numbers of total CD8+ T cells occurred at a stage when CD4+ T cell numbers had already started to drop (Figure 4.10A). Likewise, the percentage of T cells expressing CD8 was greatest late during culture, when total cell numbers were on the way down, in these three cultures. However, the extent and timing of T cell proliferation varied between different donors. The cultures from donors 12 and 13 (Figure 4.10A,B), had maximum T cell numbers at an earlier time point than a similar culture from donor 14 (Figure 4.10C). Figure 4.10 exemplifies the great variability of proliferation in different cultures. The overall extent of proliferation was much greater in the culture from donor 12 (figure 4.10A) than in the other two cultures (figure 4.10B,C).

4.2.6 IFN-γ Secretion and HLA-CMVpeptide-Tetramer Staining

4.2.6.1 *In vitro* Generated CMV-Specific T Cell Lines Secrete IFN-γ after Re-Stimulation - Optimisation of the IFN-γ Secretion Assay

Short specific stimulation with antigen presented by APC can induce antigen-specific T cells to transiently produce IFN-γ. Live, IFN-γ-positive T cells can then be labelled using the IFN-γ-secretion assay, allowing their isolation and characterisation.

However, *in vitro* antigen-driven expansion of cognate CMV-specific T cells in MoDC/T cell co-cultures for cellular therapy involves long-term and repeated restimulation by APC, usually resulting in massive proliferation of antigen-specific T cells. Since antigen-induced IFN-γ-secretion by T cells is transient, long-term specific stimulation can result in a decline in the frequency of IFN-γ-secreting T cells over time. This applied to *in vitro*-generated CMV-specific T cell lines, as illustrated by the example in figure 4.11, which shows a

reduction of IFN-γ+ cells from day 1 to day 4 of culture, as measured by the IFN-γ secretion assay without re-stimulation. Hence, the kinetics of IFN-γ expression posed limitations on the ability to detect *in vitro* expanded antigen-specific T cells by their IFN-γ production in longer-term cultures. To try to overcome these limitations, post-culture T cells were re-stimulated with antigen-pulsed APC again for the last 16h prior to the assay. The 16-h time span was chosen in accordance with recommendations in the manufacturer's instructions for re-stimulation of T cells with whole protein antigens. As shown in figure 4.12A, a restimulation with CMV antigen-pulsed DC resulted in an approximately 20-fold increase in the frequency of post-culture IFN-γ-secreting T cells when compared to re-stimulation with control antigen in this culture. In all following experiments, post-culture T cells were first cryopreserved, later thawed prior the final pre-assay 16-hour re-stimulation. Ligation of T cell-CD28 reduces activation thresholds. The effect of including an anti-CD28 mab in the post-culture re-stimulation is depicted in figure 4.12B. A greater proportion of T cells secreted IFN-γ after restimulation in the presence of anti-CD28 mab. At the start of co-culture, antigen-pulsed, CD40L-matured DC were found to be the most effective inducers of CMV-specific T cell lines capable of secreting IFN-γ (see figure 4.4). Likewise, CD40L-DC appeared to be at least as effective as iDC for induction of post-culture IFN-γ secretion at the final re-stimulation before the assay (figure 4.13A). However, CMV antigen-pulsed PBMC were sometimes found to be at least as effective as immature DC or CD40L-matured DC in the 16-hour restimulation prior to the IFN-γ-secretion assay (e.g. figure 4.13B), and a combination of MoDC and PBMC may be beneficial (figure 4.13C).

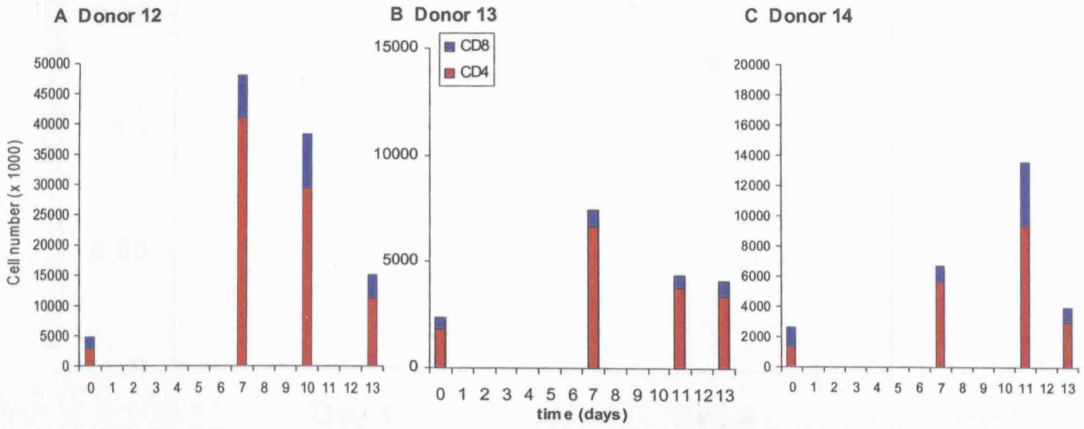

Figure 4.10 CMV Antigen-Driven T Cell Cultures: Cell Counts at Different Time Points.

Cultures were set up in triplicate for each donor. Each culture was harvested at a different time point and viable cells were counted by using Trypan blue exclusion. For each time point, a sample was stained for CD3, CD4, and CD8 expression and analysed by flow cytometry. The graphs show cell counts at different time points and the relative contribution of CD4 (red) and CD8 (blue) to the to total cell number.

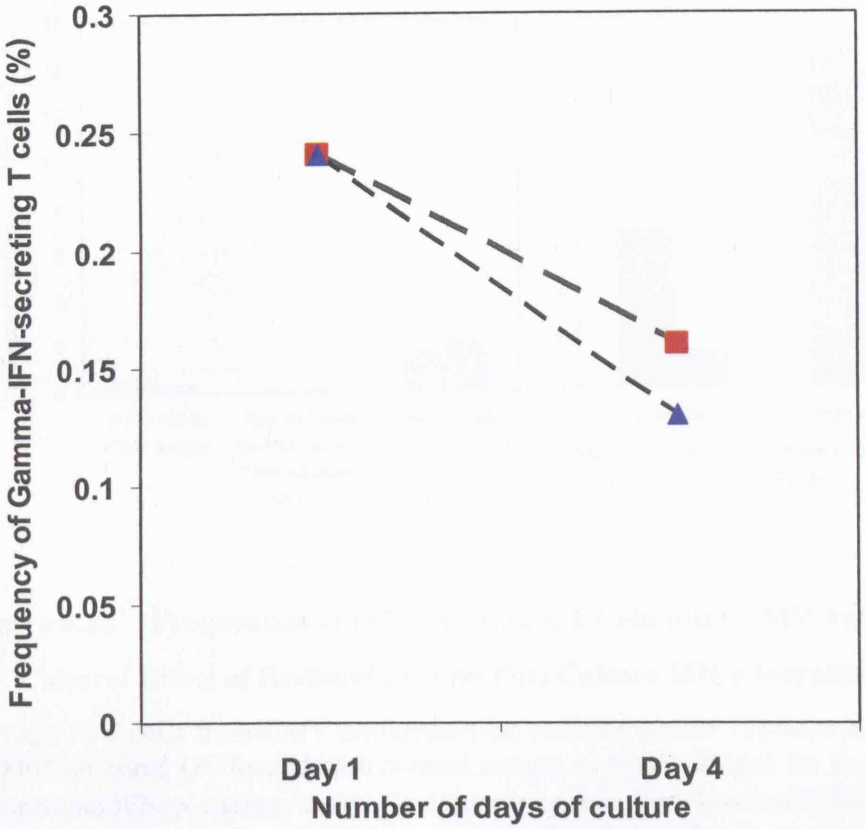

Figure 4.11 IFN-γ-Secretion by Cultured T Cells Over Time.

Co-cultures of CD40L-matured, CMV antigen-pulsed DC and autologous PBL from CMV seropositive donor 5 were set up at the same time and harvested at different time points, on days 1 and 4, for characterisation by the IFN-γ secretion assay without further restimulation. PE-labelled IFN-γ-secreting T cells where analysed by flow cytometry. **Triangles**, % of CD8+ T cells secreting IFN-γ; **Squares**, % of CD4+ T cells secreting IFN-γ.

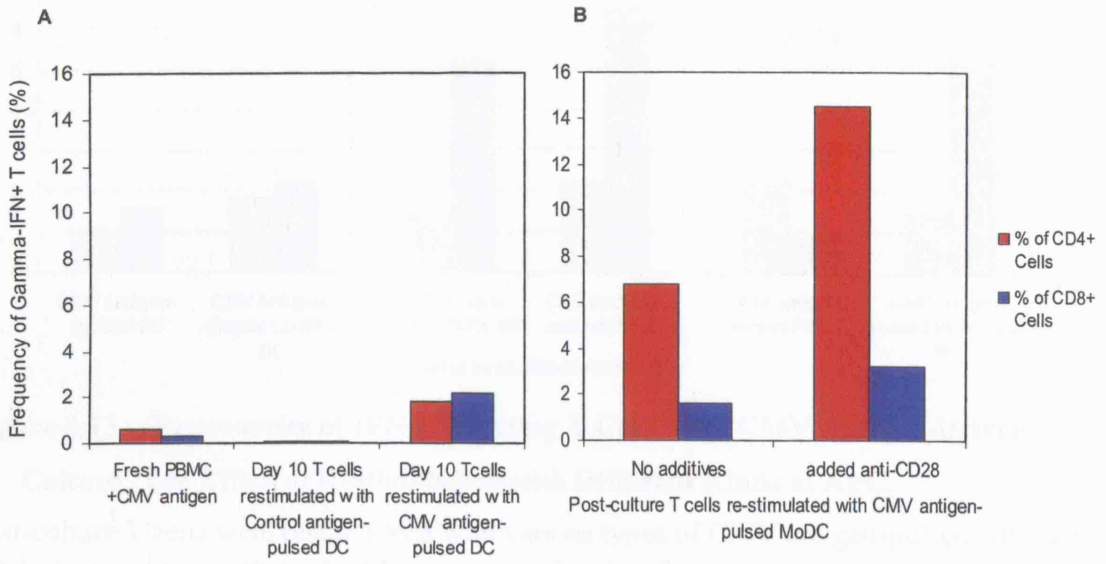

Figure 4.12 Frequencies of IFN-γ Secreting T Cells after CMV Antigen-Driven Culture: Effect of Restimulation on Post Culture IFN-γ Secretion.

A, Day 10 T cells from CMV-antigen-driven cultures (donor 13) were restimulated with CD40L-matured DC loaded with control antigen or CMV antigen for the final 16 hours prior to the IFN-γ–capture assay. To determine pre-culture levels of CMV-specific IFN-γ producing cells, uncultured PBMC were treated with CMV antigen. Fresh PBMC were treated with control antigen to establish background frequencies of IFN-γ producing cells (0.1 % of CD4+ T cells, 0.01% of CD8+ T cells) were subtracted from all other values to obtain frequencies of CMV-specific IFN-γ+ T cells. **B**, Day-11 post-culture T cells were re-stimulated with CMV antigen-pulsed MoDC, with and without anti-CD28 co-stimulation (donor 6).

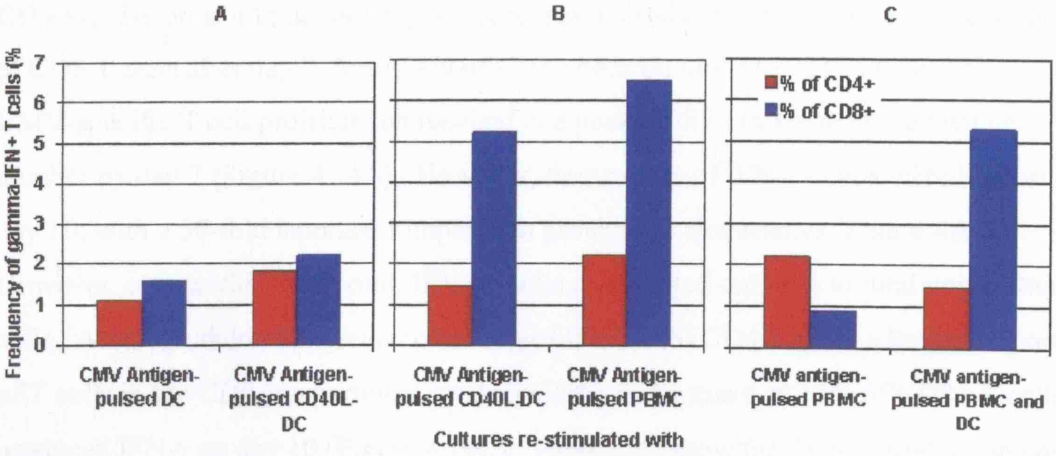

Figure 4.13 Frequencies of IFN-γ Secreting T Cells after CMV Antigen-Driven Culture: The Effect of Restimulation with Different Kinds of APC.

Post-culture T cells were restimulated with various types of CMV antigen-pulsed APC for 16h before gamma-capture. **A**, IFN-γ-secreting T cells after post-culture restimulation with CMV-antigen-pulsed immature vs mature DC (donor 5). **B**, IFN-γ-secreting T cells after post-culture restimulation with CMV antigen-pulsed mature DC vs PBMC (donor 6). **C**, IFN-γ-secreting T cells after post-culture restimulation with CMV antigen-pulsed PBMC, or CMV antigen-pulsed PBMC and DC (donor 13).

4.2.6.2 The Proportion of T Cells Secreting IFN-γ During CMV-Antigen-Driven Culture Varied at Different Stages

In an attempt to explore the relationship between T cell proliferation and the dynamics of IFN-γ secretion, triplicate cultures were set up from a single blood-draw from one CMV seropositive donor. Each culture was harvested and counted at a different stage: at a time of rapid growth (day 7), shortly after slow down in growth (day 10), and after marked reduction in the number of total viable cells (day 13). On days 6, 9, and 12 all cells remaining in culture were re-stimulated with antigen-pulsed DC, so that all cultures were restimulated at the same time, one day before each harvest point. The culture output was frozen at -80°C for later characterisation by the IFN-γ secretion assay and TCR CDR3 spectratyping.

The total lymphocyte count was highest on day 7 and started to drop some time between days 7 and 10 (Figure 4.14A). Post-culture populations of T cells were skewed towards

CD4 expression at all time points, but there was a steady increase in the relative proportion of CD8 T cells after day 7, from 14% of CD3+ cells on day 7 to 23% on day 13. CMV-specific T cell proliferation resulted in a peak 13-fold increase in the total cell number by day 7 (Figure 4.14A). However, frequencies of IFN-γ+ cells were highest on day 10, with a 50-fold increase compared to pre-culture frequencies (Figure 4A,B). However, even at this time point, IFN-γ+ cells contributed only 6% to total post-culture T cells. Although there was greater overall proliferation of CD4 T cells, a larger proportion of T cells in the CD8 compartment produced IFN-γ. As many as 15% of CD8+ T cells produced IFN-γ on day 10 (Figure 4.14C). These data show that IFN-γ-production could be induced in a substantial, yet variable, and relatively small proportion of all post-culture CMV-specific T cells.

4.2.6.3 Significant Increase in Frequencies of IFN-γ Secreting T Cells from Pre- to Post-Culture

Frequencies of IFN-γ-secreting cells in pre-culture PBMC were compared to frequencies after CMV antigen-driven T cell culture in 9 CMV seropositive donors. To take into account the varying growth rates of CMV-specific T cells in culture, cultures were maintained until one day after slow down of proliferation as indicated by less frequent requirement for media change, so that cultures were harvested at a similar stage of growth, between days 9 and 14. There was a significant increase in frequencies of IFN-γ+ T cells from pre- to post-culture in CD4 (0-1.1% pre to 1.5-18% post, (paired t-test: $p=0.024$)) and CD8 (0-2.3% pre to 1-23% post, (paired t-test: $p=0.027$) T cell compartments (Figure 4.15).

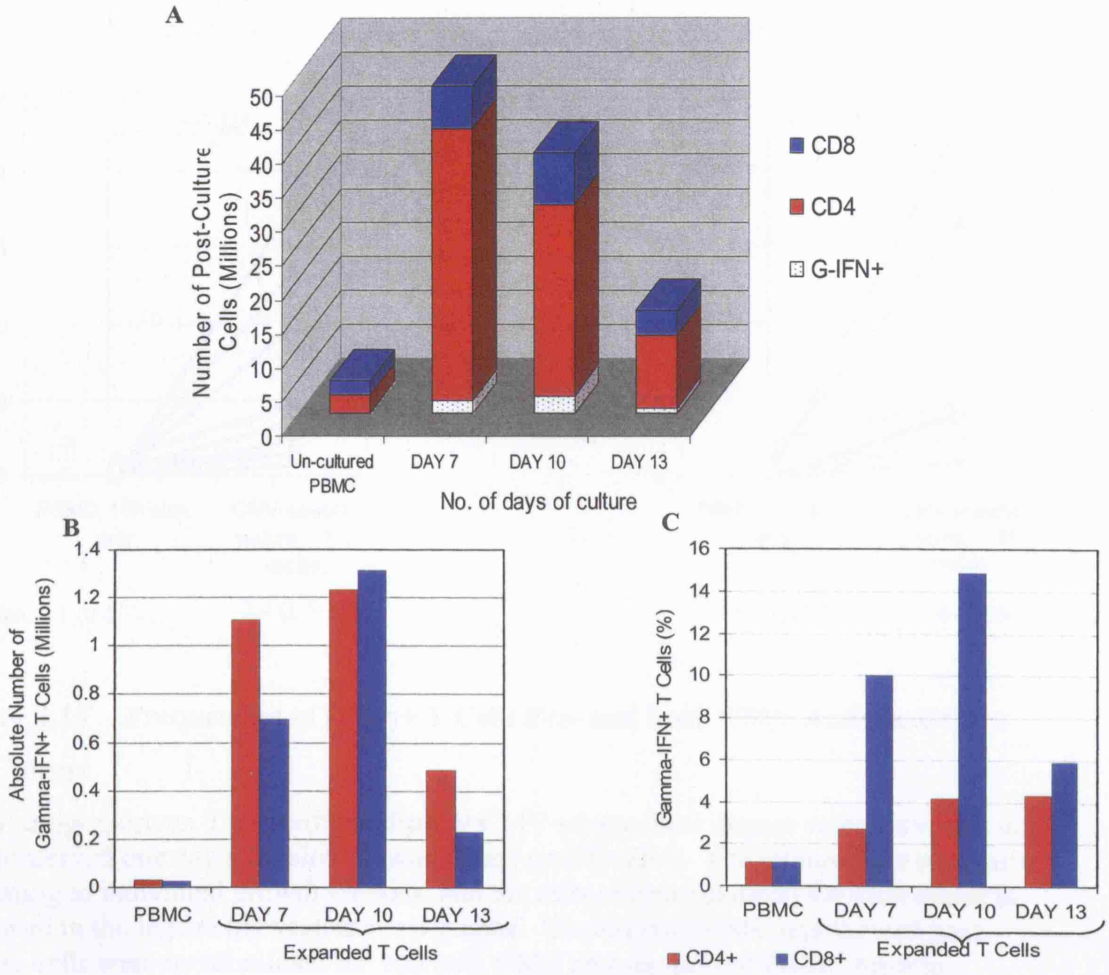

Figure 4.14 Kinetics of IFN-γ Secretion by Cultured T Cells.

CMV antigen-driven cultures from CMV seropositive donor 12 were maintained for various lengths of time before cryopreservation. Post-culture cells harvested and cryopreserved at 3 different time points were thawed, re-stimulated O/N with CMV antigen-pulsed autologous PBMC for the last 16h before analysis by the IFN-γ secretion assay. **A,** Post-culture total cell count and composition (CD4+, CD8+, and IFN-γ+ T cells); **B,** Total cell numbers of IFN-γ-secreting T cells of CD4+ (red) and CD8+ (blue) phenotype. **C,** % of CD4- and CD8-expressing T cells secreting IFN-γ.

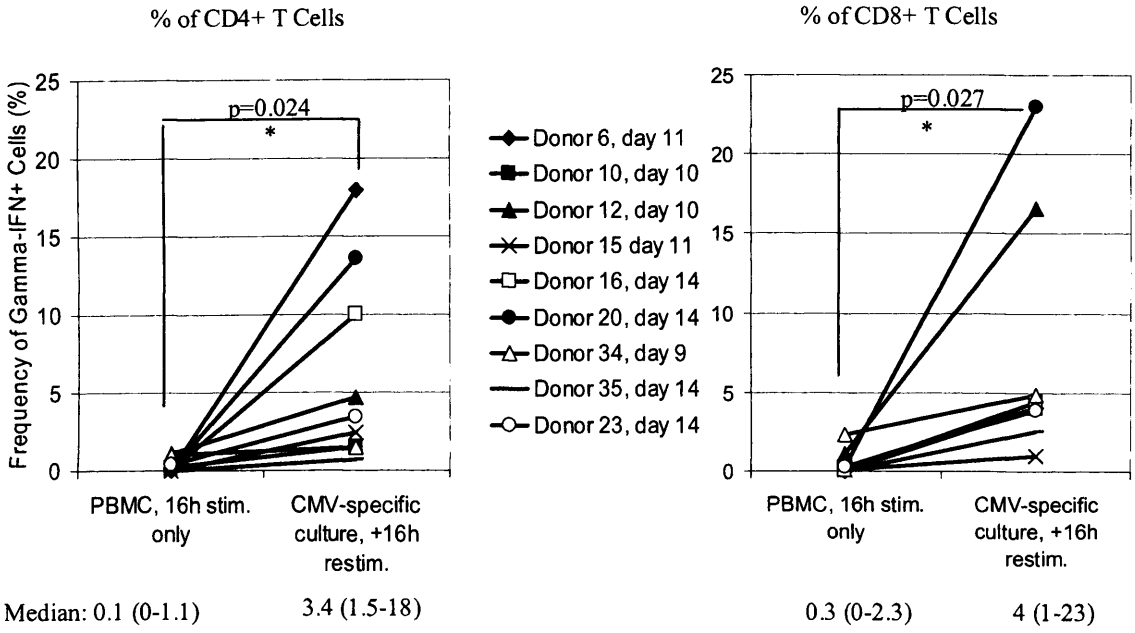

Figure 4.15 Frequencies of IFN-γ+ T Cells Pre- and Post- CMV Antigen-Driven Culture.

CMV antigen-driven T cell cultures from 9 CMV seropositive donors were harvested and cryopreserved one day after slow down in their proliferation. The culture time was varied according to individual growth kinetics, and the culture time (in days) for each donor is indicated in the legend between the two graphs. Uncultured PBMC and thawed post-culture cells were re-stimulated for 16h with CMV antigen-pulsed PBMC prior to characterisation by the IFN-γ-secretion assay. **A**, pre- and post-culture frequencies of IFN-γ-secreting T cells in the CD4 compartment. **B**, pre- and post-culture frequencies of IFN-γ-secreting T cells in the CD8 compartment. **Asterisks** indicate significant differences (paired t-test, n-2 df).

4.2.6.4 TCR CDR3 Spectratypes of IFN-γ Secreting T Cells in CMV Antigen-Driven Cultures

TCR CDR3 BV spectratyping can serve as a tool for the characterisation of post-culture CMV-specific T cells by their BV gene and size class expression.

In some donors TCR CDR3 spectratypes of total post-culture T cells did not change markedly throughout the second culture week, as shown in figure 4.16A, donor 12. However, in other cultures the CDR3 size classes dominating any given BV spectratype varied somewhat during the equivalent time period e.g. figure 4.16B, donor 14.

As shown in the example in Figure 4.17, for donor 12 the size classes expressed by the IFN-γ positive T cell subpopulation were nearly identical over time, in agreement with the lack of change in spectratype shapes of unselected post culture T cells for this donor (see figure 4.16A). This was true for both CD4 and CD8 T cell compartments. However minor size classes were not always consistently expressed throughout time. The number of sorted cells divided between the 13 spectratyping PCR reactions was close to the minimum (10 000 cells) required for reproducibility. Hence it is possible that stochastic events, which occur when cDNA from too few cells is spread between different PCR reactions required for covering all 23 BV-families, can impose limitations on comparative TCR CDR3 spectratyping.

4.2.6.5 TCR CDR3 Spectratypes of IFN-γ-Secreting T Cells were more Restricted than Those of Total Post-Culture T Cells

In 10/10 CMV seropositive donors, post-culture T cell populations expressed TCR CDR3 size classes in the full range of 23 BV families. 72- 95% of the size classes detected in pre-culture T cells were also expressed in post-culture T cells (Example: table 4.1). TCR CDR3 spectratyping also revealed a more skewed size-class distribution pattern across all 23 BV families in post-culture T cell populations compared to the near normal distribution typical for uncultured PBMC (e.g. Figure 4.16,17,18, pre- and post-culture). In most post-culture BV spectratypes one or several size classes were greatly over-represented, giving rise to very dominant peaks (Figure 4.16,17,18, post-culture).

Spectratypes of the IFN-γ-secreting subpopulation of post-culture T cells were even more skewed than those of total post-culture T cells, (example in figure 4.18) with a significantly

smaller median number of peaks/BV family in the 5/5 donors characterised (2-tailed student's T test, p=0.02) (total post-culture: median 5.1 peaks/spectratype, compared to median 3.1peaks/spectratype in the IFN-γ-secreting subpopulation) (Example: Table 4.1). The same VB-size class peaks, which dominated in unselected post-culture spectratypes, corresponded to size class peaks of IFN-γ-secreting-T cell spectratypes, as shown in the examples in Figure 4.18. Sorting IFN-γ+ cells by their CD4/8 expression revealed that some CDR3 size class fragments were expressed by either CD4+ or CD8+ T cells, whereas others were expressed by both populations (Figure 4.18).

IFN-γ+ T cells from 3/4 donors analysed were more restricted in the CD8 compared to the CD4 T cell compartment, with fewer size-class peaks covering fewer BV-families (Figure 4.18 and Table 4.1). In 1/4 donors the opposite was true.

T Cells	Total Number of Peaks	Number of BV-Families
Pre-Culture PBMC	167	23/23
Post-Culture CMV-specific	116	23/23
Post-Culture IFN-γ+	79	23/23
Post-Culture IFN-γ+/CD4+	59	19/23
Post-Culture IFN-γ+/CD8+	44	15/23

Table 4.1 TCR CDR3 BV-families and size classes expressed by CMV-specific- and uncultured T cell populations.

RNA was extracted from PBMC, post-culture CMV-specific T cells, MACS- and flow cytometry-sorted post-culture IFN-γ secreting T cells. The RNA was analysed by TCR CDR3 spectratyping. The table displays number of total size-classes expresses across all 23 BV families, and number of CDR3 BV families expressed by the various T cell populations from CMV seropositive donor 15.

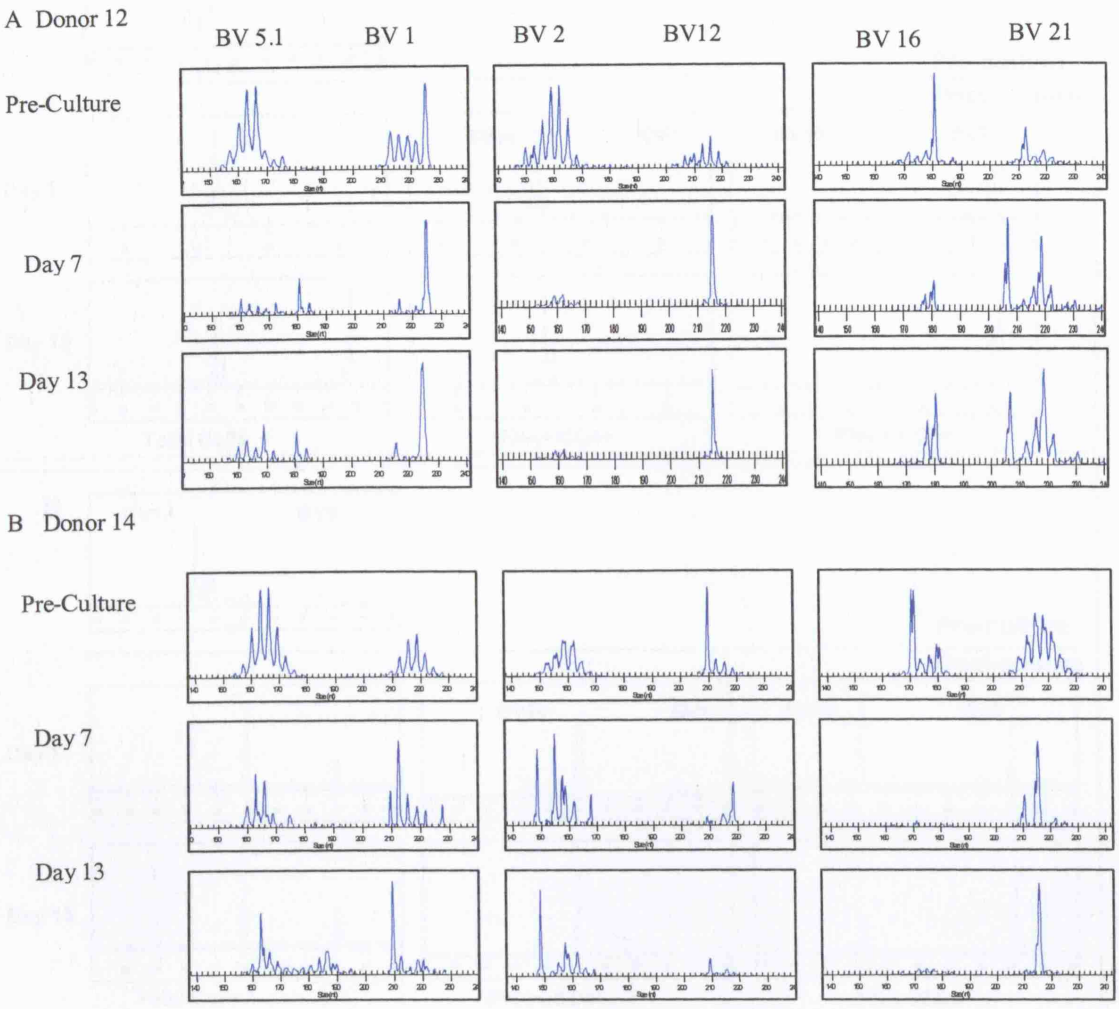

Figure 4.16 TCR CDR3 Spectratypes over Time in CMV Antigen-Driven T Cell Cultures.

The panels show TCR CDR3 spectratypes of 6 BV families for CMV antigen-driven cultures from 2 CMV seropositive donors at 3 different time points: pre-culture, culture day 7, and culture day 13. **A)** donor 12: Example for spectratypes not changing over time. The same size classes dominate in TCR BV spectratypes between days 7 and 13 of CMV antigen-driven culture. **B)** Donor 14: Examples of spectratypes that changed over time. Different TCR BV size classes dominate spectratypes on days 7 and 13 of culture.

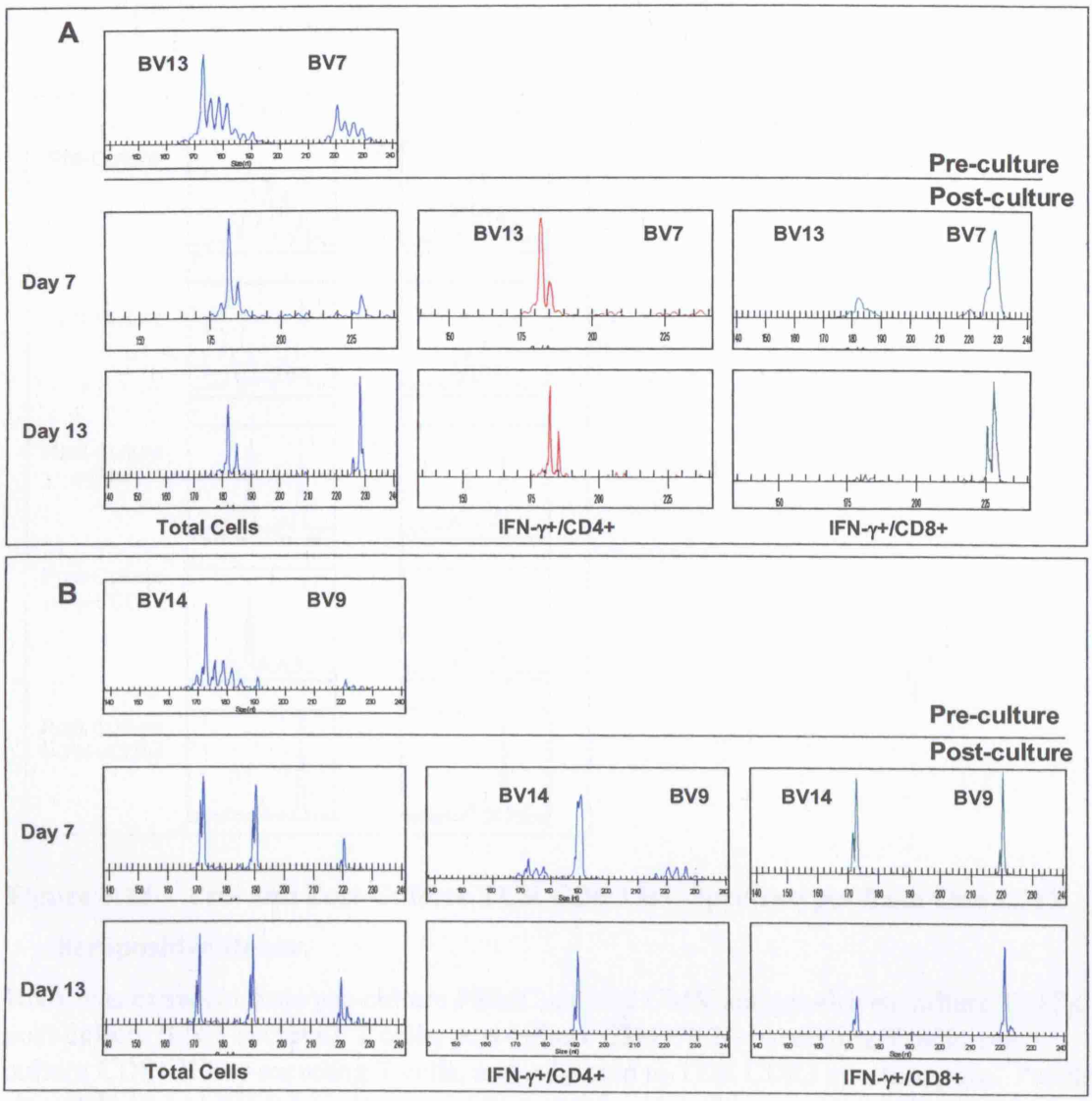

Figure 4.17 TCR CDR3 Spectratypes of Post-Culture T Cells over Time.

Parallel CMV antigen-driven T cell cultures from CMV seropositive donor 12 were harvested on days 7, 10, and 13, respectively. IFN-γ-secreting T cells were sorted by flow cytometry from thawed, re-stimulated post-culture cells. RNA from pre-culture, post-culture, IFN-γ+/CD4+, and IFN-γ+/CD8+ post-culture T cells was analysed by TCR CDR3 spectratyping. The panels show products of duplex RT PCRs from day 7 and 13 cultures. **A**, BV13 and BV17; **B**, BV14 and BV9. Spectratypes for day10 cultures were nearly identical (data not shown).

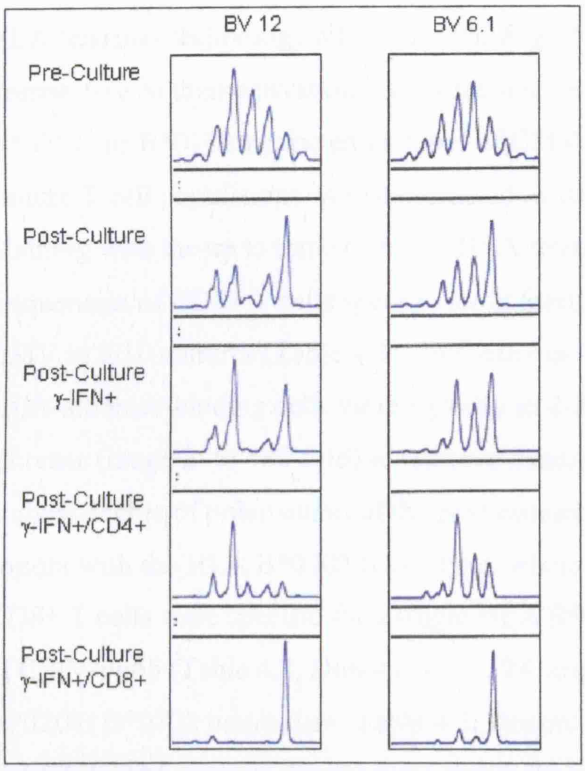

Figure 4.18 Pre- and Post-Culture TCR CDR3 BV Spectratypes from One CMV Seropositive Donor.

RNA was extracted from pre-culture PBMC, day-12 CMV antigen-driven culture T cells, post-culture IFN-γ-secreting T cells, post-culture CD4+/IFN-γ-secreting T cells, post-culture CD8+/IFN-γ-secreting T cells, and subjected to TCR CDR3 spectratyping. Panels show BV 12 and BV 6.1 spectratypes (donor 15).

4.2.6.6 T Cells Specific for Single Immunodominant Peptides of CMV Increase in Number from Pre-to Post-Culture

HLA-tetramer technology allows the staining of T cells specific for single epitopes, irrespective of their activation state. Frequencies of CD8+ T cells specific for HLA A*0201- or B*0702-restricted epitopes of CMV pp65- or IE-1 proteins in pre- and post-culture T cell populations were determined in 10 donors.

Staining with the up to three different HLA-tetramers revealed an increase in the frequencies of CD8+ T cells specific for at least one of three immunodominant epitopes of CMV in 9/10 cultures (Table 4.2). In these nine cultures, the extent of proliferation of HLA-tetramer-binding cells varied greatly in different donors, with a median 10-fold increase (range 2- to 480-fold) in tetramer-binding T cells from pre- to post-culture. The highest degree of polarisation of the post-culture CD8 T cell response was observed in donors with the HLA B*0702 tissue type, where up to 53% (Figure 4.19) of all post-culture CD8+ T cells were specific for a single HLA B*0702-restricted epitope (TPRVGGGAM) of CMV-pp65 (Table 4.2, Donors 16, 17, 24, and 25). In the two donors with HLA A*0201/ B*0702 tissue type (Table 4.2, Donors 24, 25), the numbers of HLA A*0201 restricted pp65-specific T cells were markedly lower than the HLA B*0702 restricted cells. A 4-fold increase in the frequency of T cells specific for a newly identified HLA A*0201-restricted epitope of IE-1 (VLEETSVML) from pre- to post-culture was detected in 1/3 donors. In this donor, post-culture frequencies of HLA A*0201-restricted $pp65_{NLVPMVATV}$ -specific T cells had also increased 3-fold (Table 4.2, Donor 23). The *in vitro* CMV-specific proliferative T cell response was directed at epitopes from a minimum of two different antigens of CMV in this donor.

	HLA A*0201 $pp65_{NLVPMVATV}$-binding T Cells			HLA A*0201 $IE1_{VLEETSVML}$-binding T Cells			HLA B*0702 $pp65_{TPRVTGGGAM}$-binding T Cells		
	Pre-Culture	*Post-Culture*		*Pre-Culture*	*Post-Culture*		*Pre-Culture*	*Post-Culture*	
Donor (HLA-Type)	(% of CD8+)	(% of CD8+)	Fold-expansion	(% of CD8+)	(% of CD8+)	Fold-expansion	(% of CD8+)	(% of CD8+)	Fold-expansion
16 (B*0702)	NA	NA	NA	NA	NA	NA	0.5	31	103
17 (B*0702)	NA	NA	NA	NA	NA	NA	1.4	2.4	*10*
18 (A*0201)	0.34	3.5	*15*	ND	ND	NA	NA	NA	NA
19 (A*0201)	0.22	0.6	*5*	ND	ND	ND	NA	NA	NA
20 (A*0201)	0.03	1.4	*480*	ND	ND	ND	NA	NA	NA
21 (A*0201)	0.34	3.5	*15*	ND	ND	ND	NA	NA	NA
22 (A*0201)	1.3	1.6	*0.75*	0.8	0.7	NA	NA	NA	NA
23 (A*0201)	2.5	7	*3*	3	12	4	NA	NA	NA
24 (A*0201, B*0702)	1.2	0.6	NA	ND	ND	ND	1.1	4.4	*2*
25(A*0201, B*0702)	1.1	1.9	*4*	0.7	0.4	NA	1.6	53	*6*

Table 4.2 Pre- and Post-Culture Frequencies of CD8+ T Cells Binding HLA-Tetramers.

PBMC and T cells from CMV-antigen-driven cultures were stained with HLA A*0201-$pp65_{NLVPMVATV}$ -tetramers, HLA A*0201 $IE1_{VLEETSVML}$-tetramers, or HLA B*0702-$pp65_{TPRVGGGAM}$ -tetramers conjugated to PE according to donor tissue type, then co-stained with anti-CD8-FITC mab. Cells were then analyzed by flow cytometry. The table shows the percentage of CD8+ T cells binding to each HLA-tetramer. Where applicable, fold-expansion of tetramer+ T cells from pre- to post-culture is also displayed. (NA) not applicable, (ND) not done.

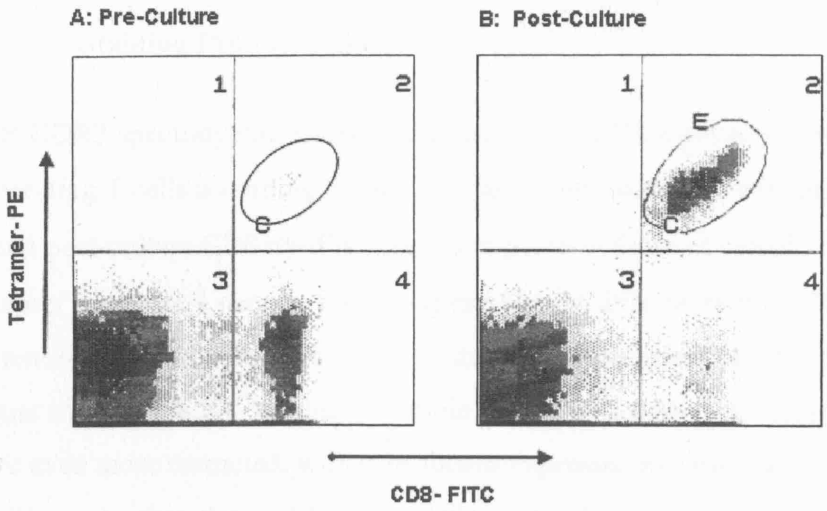

Figure 4.19 Pre- and Post-Culture Levels of HLA CMVpeptide-Tetramer-Binding T Cells.

Flow diagrams of T cells stained with anti-CD8-FITC and HLA B*0702 pp65TPRVTGGGAM-tetramer-PE. **A**, of PBMC; **B,** T cells from a day-14 CMV-antigen-driven culture from CMV seropositive donor 16.

4.2.6.7 Not all CMV-Specific T Cell Clones Identified by HLA-$CMV_{peptide}$-Tetramer Staining Produced IFN-γ

TCR CDR3 spectratyping allows the comparison of HLA-tetramer binding T cells to IFN-γ-secreting T cells according to their BV family and size class expression. Spectratypes of sorted post-culture CD8+/γ-IFN+ were compared to those of sorted HLA-$CMV_{peptide}$-tetramer + cells in 4 donors. Spectratypes of both γ-IFN-secreting CD8+ T cells and HLA-tetramer-binding T cells were highly restricted, with expression of very few BV size classes in just some of the BV families (example in figure 4.20A). Spectratypes of tetramer+ cells were even more restricted, with size classes expressed in a median of 4/23 (range 3-8) BV-families only, than those of IFN-γ+/CD8+ post-culture T cells, which expressed size classes in a median of 14/23 (range 9-20) BV-families (Figure 4.20B). A median of 50% (range 0-78%) of size classes expressed by HLA-tetramer-binding T cells were also expressed by IFN-γ+/CD8+ T cells from the same donor (Figure 4.20B). And vice versa, a median of 9% (range 0-40%) of size classes expressed by IFN-γ+/CD8+ T cells were also expressed by tetramer-$CMV_{peptide}$-binding cells (Figure 4.20B). Shared peaks may have originated from expansion of the same T cell clones, i.e. CMV-specific tetramer-binding cells which could be re-activated to produce IFN-γ. The remaining size classes were unique to either tet+ cells or IFN-γ+/CD8+ T cells. This indicated the existence of CMV-specific IFN-γ responses by T cells either restricted by another HLA allele, or specific for different peptides, or a combination of both. VB size class expression specific for HLA-tetramer-binding cells showed that not all pp65 peptide-specific T cells could readily be activated to produce IFN-γ.

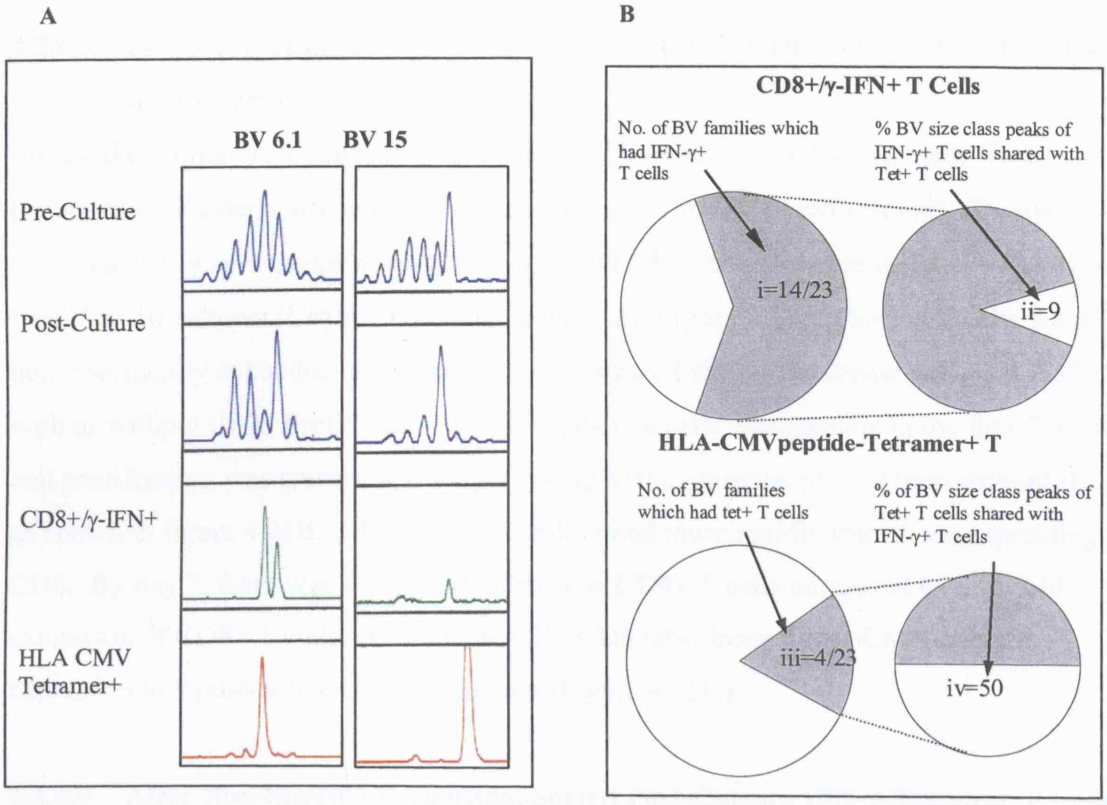

Figure 4.20 Comparison of Post-Culture TCR CDR3 Spectratypes: γ-IFN+/CD8+ vs HLA-CMVpeptide-Tetramer+ T Cells.

A, BV6.1 and BV15 CDR3 spectratypes of pre-culture, total post culture, post-culture γ-IFN+/ CD8+, post-culture HLA-CMVpeptide-tetramer+ T cells from CMV seropositive donor 12. **B**, median % total BV families and size classes expressed by different sub-populations of post-culture T cells from 4 CMV-seropositive donors: (i) median number BV families (total 23) expressed by post-culture CD8+/IFN-γ+ T cells. (ii) median % of total BV-size classes expressed by CD8+/IFN-γ+-T cell that were also expressed by HLA CMVpeptide-tetramer-binding T cells. (iii) median number of total CDR3 BV families expressed by HLA-CMVpeptide-tetramer-binding T cells. (iv) median % of total HLA-CMVpeptide-tetramer-binding T cell spectratype peaks also expressed by CD8+/IFN-γ+ T cells.

4.2.6.8 IFN-γ-Secreting T Cells from CMV Antigen-Driven Cultures Proliferated in the Presence of Growth Factors

To test the proliferative capacity of post-culture IFN-γ-secreting T cells, these were expanded non-specifically with feeder cells and recombinant growth factors. T cells secreting IFN-γ in response to re-stimulation with CMV antigen were isolated by MACS from day-10 cultures (CMV seropositive donor 12) (Figure 4.21A). Sorted T cells were non-specifically expanded in the presence of irradiated allogeneic feeder cells and IL-2, with or without IL-7. Proliferation of IFN-γ positive cells was greatest in the first 7 days. T cell proliferation was greater, and longer lasting in the presence of IL-7 than without IL-7, as shown in figure 4.21B. CD4+ T cells proliferated more rapidly than those expressing CD8. By day 7, there was a 415-fold increase in CD4+ T cells compared to a 45-fold expansion of CD8+ T cells, skewing the CD4:CD8 ratio from 1 post-CMV-specific expansion to 9 post-non-specific expansion (Figure 4.21C).

4.2.6.9 After Non-Specific Expansion, Sorted Post-Culture IFN-γ-Secreting T Cells Produced IFN-γ Again when Re-Stimulated with CMV Antigen

After 7 days, T cells expanded non-specifically using cytokines and feeder cells were re-stimulated with CMV antigen or control antigen. As shown in figure 4.19D, 50% of post-expansion T cells produced IFN-γ when re-challenged with CMV antigen, compared to only 3% when re-stimulated with control antigen.

4.2.6.10 Post-Antigen-Driven Culture- and Non-Specifically Expanded IFN-γ-Secreting Post-Culture T Cells Expressed the same TCR CDR3 BV Families and Size Classes

Figure 4.22 shows examples of VB size class usage by T cells directly after CMV-specific culture and following non-specific expansion. Overall, there was an overlap of BV size-class usage of 83% across all BV families.

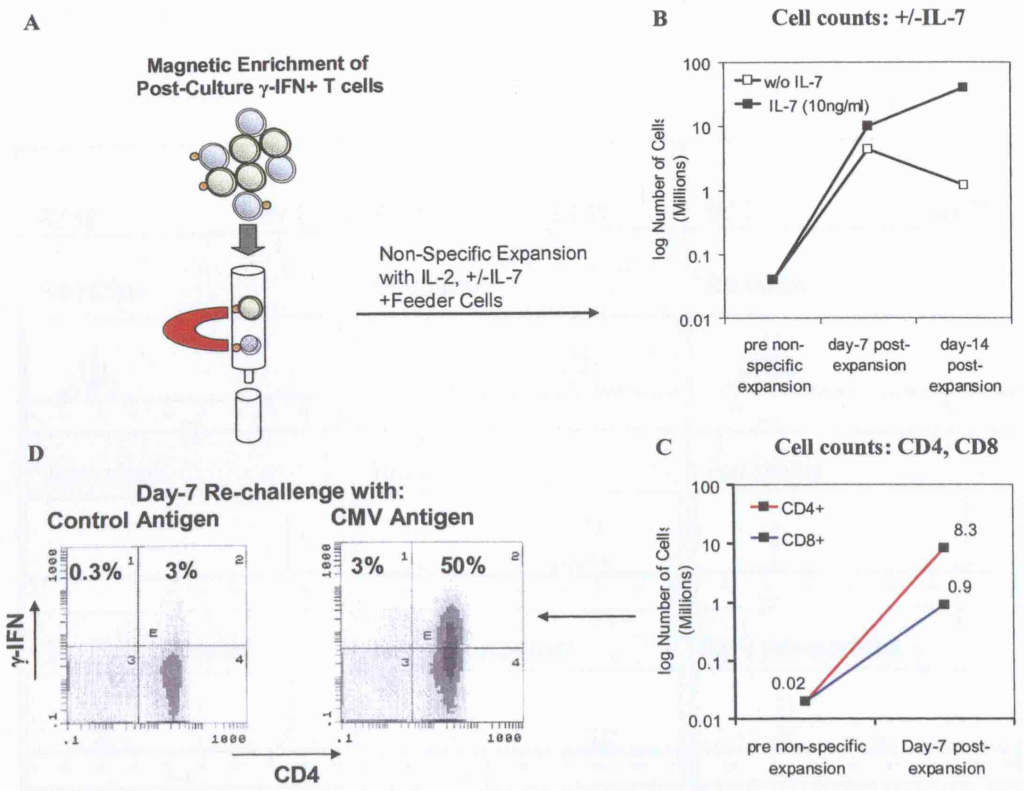

Figure 4.21 Non-Antigen-Specific Expansion of Post-Culture IFN-γ-Secreting T Cells.

A, T cells secreting IFN-γ in response to CMV antigen were isolated from day-10 CMV antigen-driven cultures by MACS (donor 12). Sorted cells were set up in duplicate cultures with irradiated allogeneic feeder cells and 50 U/ml IL-2, with or without IL-7 (10ng/ml). **B,** T cell counts: change in cell number from over 2 weeks, with and without IL-7. **C,** pre- and day-7 post-expansion counts of CD4+ and CD8+ T cells in IL-7 containing cultures. **D**, following 7 days of non-specific expansion, T cells were re-stimulated with antigen-pulsed PBMC and characterised using the IFN-secretion assay.

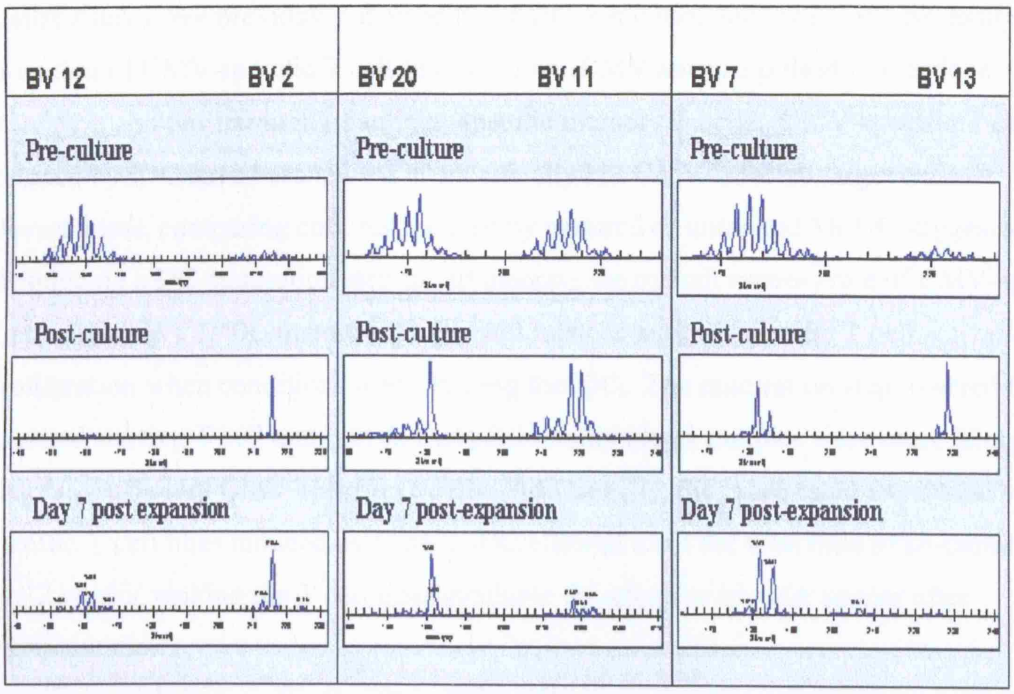

Figure 4.22 TCR CDR3 Spectratypes of Pre-Culture, Post-Culture, and Non-Specifically Expanded Post-Culture T Cells.

IFN-γ-secreting T cells isolated from a day-10 CMV antigen-driven culture by MACS were re-cultured with irradiated allogeneic PBMC in the presence of IL-2 (50U/ml) and IL-7 (10ng/ml) for 7 days. RNA was isolated from pre-culture, post-antigen-driven culture, and post non-specifically expanded T cells and reverse transcribed. Panels show spectratypes of 6 BV families generated in duplex PCR reactions.

4.3 Discussion

Following the identification of CD40L as a potential DC maturation factor for the *in vitro* induction of CMV-specific T cell lines (see Chapter 3) CD40L-DC were tested in clinical scale cultures. We previously showed that a DC maturation step was not a necessity for the induction of CMV-specific T cell lines *in vitro*. CMV antigen-pulsed iDC induce activation and proliferation of antigen-specific memory T cells. CMV-specific T cell lines induced by iDC have been shown to specifically kill CMV-infected target cells [56]. Nevertheless, comparing cultures induced by matured or untreated MoDC suggested that introducing a DC maturation step might increase the overall success rate of CMV-specific T cell cultures. CD40L-matured MoDC still induced antigen-specific T cell activation and proliferation when conditions were limiting for iDC. The maturation step lowered the required starting T cell number, so that poor quality blood samples were more likely to still generate sufficient CMV-specific T cells. Additionally, the more rapid expansion of CMV-specific T cell lines induced by CD40L-DC allowed a cut the total time of co-culture from 3 to 2 weeks, making the T cell lines available for adoptive transfer sooner after transplantation.

The conserved shapes of post-culture TCR CDR3 spectratypes seemed to suggest that CD40L-DC and untreated DC induced expansion in the same T cell clones. In addition, a greater number of T cells induced by CD40L-DC produced the TH-1-type cytokine IFN-γ. Since T cells of the TH-1 pathway, especially CD8+ CTL effectors, are strongly associated with immunity against CMV, more of the T cells induced by CD40L-DC may be of the desired phenotype.

Before the DC maturation step was included into the protocol for generation of CMV-specific T cell lines, CMV antigen had been routinely added to MoDC cultures at the time of co-culture set up together with autologous T cells, so that soluble antigen was present in the co-cultures throughout. The inhibition of T cell proliferation shown in some antigen presentation assays in the presence of soluble CMV antigen suggested, however, that levels of toxicity varied between different batches of CMV antigen. To avoid the problem of antigen-mediated inhibition of T cell proliferation, antigen can be removed from the supernatant by washing before addition of T cells to the antigen-presenting DC.

The introduction of the maturation step into the protocol necessitated a change in the timing of addition of CMV antigen to DC. Immature DC are most efficient at ingesting and breaking down antigens for HLA loading in late endosomes. Only upon DC activation do peptide-loaded HLA molecules get targeted to the plasma membrane ready for antigen presentation to T cells. Consequently, for optimal antigen uptake, pulsing DC with CMV antigen was brought forward. CD40L was routinely added to differentiating MoDC on day 4, and CMV antigen was added a minimum of 3h earlier. CMV antigen was present in cultures of differentiating MoDC until removed along with the cytokines just before the addition of autologous lymphocytes. The minimum length of exposure to CMV antigen for optimal uptake by DC has yet to be determined experimentally.
Preliminary experiments have shown that a shortening of antigen exposure reduced antigen toxicity, but higher antigen concentrations were then required for the induction of comparable T cell responses.

After a 7-day differentiation with GM-CSF and IL-4, DC cultures derived from the adherent fraction of PBMC contain adherent, lightly-adherent, and non-adherent cells. They vary in proportions across different cultures. Non-adherent and lightly-adherent cells are easily harvested by washing. Adherent cells can be harvested using a cell scraper. Non-/lightly -adherent DC were shown here to be much more potent inducers of antigen-specific T cell proliferation than adherent cells from the same culture, so that harvesting of the adherent fraction for culture re-stimulation was regarded obsolete.

Availability of DC is often the limiting factor for the maintenance of antigen-driven co-cultures over any length of time. DC numbers tend to decrease with increasing T cell activation in a culture, because T cells will specifically lyse cells presenting the antigen to which they were raised. When CMV-specific T cell lines are generated from a single blood draw, a cryopreservation step is required to make day-7 MoDC available at the time of co-culture re-stimulation. DC generated from fresh PBMC, cryopreserved and thawed, gave a greater yield than DC generated from frozen then thawed PBMC. Thawed DC derived from fresh PBMC were more likely to provide sufficient cell numbers and these were used in the amended cellular therapy protocol.

The IFN-γ secretion assay and tetramer staining were employed in combination with comparative TCR CDR3 spectratyping for the more detailed characterisation of the composition of culture output.

The IFN-γ secretion assay has great potential in a clinical setting for the isolation of live antigen-specific T cells from fresh PBMC or whole blood after a brief antigenic stimulation, which is long enough to elicit a cytokine response, but insufficient to allow the onset of proliferation[58;131]. HLA tetramer-selected cells provide an alternative source of CMV-specific CD8+ T cells. Here we explored the potential of both technologies for the isolation and characterisation of antigen-specific T cells after their expansion in culture. During culture with CMV-lysate- pulsed MoDC, T cells are continually exposed to the full range of CMV antigen-epitopes. T cell activation thresholds differ for different epitopes[132] and may also be altered after repeated re-stimulation over time. In fresh PBMC, IFN-γ production by murine T cells after activation has been shown to peak two days after stimulation with the superantigen SEB and to decline thereafter [133]. To try to overcome the limitations set by the kinetics of IFN-γ secretion, the cultures were re-stimulated with fresh antigen-presenting cells in the final 16h before the assay for maximum IFN-γ secretion. Sensitivity was further increased by the addition of co-stimulatory anti-CD28 mab to lower T cell activation thresholds.

Total number and relative frequency of IFN-γ-producing cells T cells were significantly greater in antigen-driven co-cultures than uncultured T cells after re-stimulation. Both CD8+ and CD4+ T cells secreted IFN-γ. Although direct killing of CMV infected cells by T cells generated in this culture system has previously been shown to be mediated exclusively by CD8+ T cells [56], proliferation of memory CD8 T cells has been shown to require CD4+ T cell help *in vitro*.[134] The presence of activated, IFN-γ-secreting CD4+ T cells was of interest; especially since CD4+ T cells usually outnumber CD8+ T cells after culture. CD8+ CTL alone are not sufficient for the maintenance of CMV-specific immunity[127]. In CMV seronegative individuals becoming infected with CMV, activated CD4+ T cells have a role in targeting adaptive effector- and innate responses to sites of infection, and also eventually give rise to central memory T cells after viral latency is

established[8]. IFN-γ-secreting T cells are committed to the TH1 phenotype[128]. IFN-γ plays an important role in the induction of cellular antiviral proteins[135], the activation of macrophages[136], and DC maturation[111]. All of these functions would be required *in vivo* for the development of sustained immunity in immunocompromised SCT/BMT-recipients.

The kinetics of cell proliferation and death are important determinants of the optimal timing of cell harvest in *in vitro* culture systems. In general, a few days after start of co-cultures exponential T cell proliferation commences, indicated by the requirement for frequent media change. Usually some time into the second week of culture, proliferation slows down; medium needs to be replenished less frequently. Eventually, despite addition of IL-2, large numbers of T cells apoptose. The exact timing of these events is variable between different donors. Frequency and number of IFN-γ+ T cells varied between different growth phases of the cultures, and the kinetics of IFN-γ production differed in CD4 and CD8 T cell compartments. For a maximum IFN-γ response, especially by CD8 T cells, cultures may need to be harvested just after slow down in proliferation. The introduced reduction of the standard culture time span from 3 to 2 weeks brings cell harvest closer to this time point. At any time point, only a fraction of all post-culture T cells could be restimulated to secrete IFN-γ. The majority of the T cells that proliferated in culture in response to stimulation with CMV antigen did not produce IFN-γ after re-stimulation. These may have a higher activation threshold, which could have been altered as a result of extended culture, or produce other cytokines. IFN-γ secretion is an early event in T cell activation, which occurs during their interaction with APC. IFN-γ produced by T cells can act as a second signal inducing DC maturation, which ultimately facilitates even greater T cell activation. Hence T cells that have stopped secreting IFN-γ may be further down the line of differentiation, and may still have cytotoxic effector function. Some of these cells may be represented by the population identified by CMV-specific tetramer binding that failed to secrete IFN-γ. TCR CDR3 spectratypes of normal PB T cells typically consist of a range of 7-10 different size classes for each of the 23 BV subfamilies. These are separated by multiples of 3 base pairs, because T cells expressing out of frame rearrangements are deleted in the thymus before reaching peripheral blood. Size classes near the centre of the range are usually

expressed by more T cells than those at the outer limits, so that the histogram of size class expression tends to resemble a normal distribution pattern. This is even more clear-cut in cord blood T cell spectratypes, with limited prior exposure to exogenous antigen[137;138]. Over-representation of one or several size classes within a spectratype can indicate expansion of T cell clones due to challenge with antigen. Compared to pre-culture, CDR3 spectratype patterns of post-culture T cells were much more skewed. Spectratypes of CMV antigen-driven T cell cultures consisted of some very predominant peaks in the individual BV-families. Characterisation of the culture output at various time points during the second half of the culture period showed conserved CDR3 spectratype patterns for some, and changes in spectratype patterns for others. This variability may be due to different growth rates of cultures, perhaps reflecting variations in precursor numbers and in the immune status of the donor at the time of the blood draw. Furthermore, dominant size class peaks did not differ markedly between spectratypes of post-culture/IFN-γ+ cells and total post-culture T cells. This strongly suggests that cells derived from the same expanded T cell clone produced IFN-γ at the various stages of culture. This may not reflect the *in vivo* situation during CMV infection, because CMV antigens exposed to the immune system vary during the viral life cycle. The relatively large number of different clones shown by spectratyping to have been expanded in post-culture populations indicates the broad antigenic-specificity of the culture output. In immunotherapy this may be advantageous because it reduces the likelihood of immune evasion by the virus.

Until recently, the protective anti-CMV CD8 T cell response in humans had been considered to be directed largely towards a single antigen: the lower matrix protein, CMV-pp65 [65;139;140]. Furthermore, there was evidence that in the HLA A*0201 restricted response the bulk of pp65-specific T cells are specific for a single nonamer peptide [140]. However, the limitation of experiments largely to a single laboratory strain of CMV may have resulted in underestimation of the *in vivo* role of other CMV antigens e.g. IE-I [70;129;130]. *In vivo*, different peptide epitopes may be targeted at different stages of the immune response [70]. Some responses may be missed if single peptides of e.g. pp65 are used to stimulate cytokine secretion prior to cell selection, as has been proposed for some clinical studies. The highly focused HLA B*0702-restricted *in vitro* response in one donor is an example of

how polarised anti-CMV responses can be. The combination of donor HLA-alleles may influence the contribution of T cells specific for individual epitopes to the overall anti-CMV response. For example, B*0702-restricted pp65-specific T cell numbers by far outnumbered A*0201-restricted T cells in donors expressing both alleles, a phenomenon observed by others[141]. Other combinations of HLA molecules are likely to skew T cell responses in different ways, thus affecting the proportion of CMV-specific T cells that can be detected by staining with single HLA-tetramers. In practice, using a limited number of different HLA-tetramers to isolate CMV-specific T cells, makes missing significant CD8 responses directed against other epitopes and/or restricted by another HLA allele likely.

Comparison of TCR CDR3 spectratypes of IFN-γ+/CD8+ T cells to those of HLA-tetramer+ T cells indicates that selection based on either technique will exclude a significant population of CMV-specific T cells. Spectratypes of HLA-tetramer+ post-culture T cells consisted of a smaller repertoire of BV size classes than IFN-γ/CD8+ post-culture T cell spectratypes, indicating that the overall CD8+ response was directed against a greater range of epitopes of CMV antigen, restricted by the same or other HLA alleles. In addition, not all CMV-specific (HLA-$CMV_{peptide}$-tetramer+) cells could be activated to produce IFN-γ, and it has previously been shown that HLA-tetramer+ T cells were not always fully functional.[142]

Because cells intended for adoptive transfers are not labelled, monitoring the fate of CMV-specific T cell lines after infusion relies on indirect evidence, such as detecting the same tetramer-binding CMV-specific T cells in post-culture as in post-infusion blood samples. However, the origin of such T cells remains unclear. T cells binding specific HLA-tetrameric complexes in post infusion blood samples could originate from donor T cells that were already present in the initial transplant, from T cells reconstituted from graft stem cells, or from the infused CMV-specific T cell lines.
It has previously been shown that post-culture cells were capable of specific, HLA class I-mediated lysis [143]. To reduce the risk of alloreactivity only a very small dose of post-culture T cells is used for re-infusion[46], and post-infusion CD8+ T cell re-activation and proliferation is likely to be necessary for the induction of sufficient lysis of CMV-infected

cells *in vivo*. In addition, for the control of infection in the longer-term, CD4-mediated immunological memory would need to be established. Showing that a proportion of post-culture CD4 and CD8- T cells are capable of becoming re-activated to produce IFN-γ, and that such IFN-γ-secreting T cells have the required further proliferative potential is important in this context.

CHAPTER 5

Attempts at Specific T Cell Expansion in CMV Seronegative Donors

5.1 Introduction

In vivo, mature DC migrating from sites of infection are capable of inducing primary T cell responses in the lymph nodes. By manipulating *in vitro* generated MoDC maturation we tried to activate naïve, CMV antigen-specific T cells *in vitro*.

IL-15 is a T cell growth factor, which has many properties in common with IL-2 and IL-7. Both IL-15 and IL-7 have a role in the homeostatic maintenance of T cell populations in the blood, acting as survival signals, and inducing non-antigen-dependent proliferation when the T cell pool is depleted. IL-15 is also produced by activated DC, enhancing their immunostimulatory ability [105]. Although naïve T cells can be primed with IL-7 [144], or IL-15 [145] prior to co-culture with APC to lower their activation threshold, this has not been successful in the CMV culture system in our hands. Alternatively, we looked at the effect of IL-15 when added to cultures at a later stage.

Activated APC can produce IL-12 which helps T cell activation and skews T cell responses towards the TH1 pattern of cytokine production [123]. Here, antigen-driven cultures were exposed to immunostimulatory cytokines during culture, to specifically try to stimulate those T cells already in contact with APC.

5.2 Results

5.2.1 Monitoring of CMV-Specific T Cell Proliferation by ^{3}H-Thymidine Incorporation

5.2.1.1 MoDC *in vitro* Matured by Various Methods Induced Proliferation of Naïve CMV-Specific T Cells only on Rare Occasions

As described in chapter 3, various mediators of DC maturation were tested in an attempt to generate the most effective APC for the *in vitro* generation of CMV-specific T cell lines from CMV seropositive donors for adoptive cellular therapy. MoDC exposed to CD40L, LIGHT, TNF-α and PGE_2, or the cocktail of TNF-α, IL-1, IL-6, and PGE_2 all showed some enhancement in their capacity to induce *in vitro* T cell proliferation in CMV seropositive donors compared to immature DC. These agents were then evaluated for CMV seronegative donors. The diagrams in Figure 5.1 show antigen presentation/T cell proliferation assay results from two CMV seronegative donors, using MoDC matured with various maturation-inducing mediators as APC, co-cultured with autologous lymphocytes. MoDC, matured with TNF-α alone (Figure 5.1A (donor 2), a cocktail of TNF-α, IL-1, PGE_2 (Figure 5.1B (donor 2)), CD40L alone, or in combination with PGE_2 or LIGHT (Figure 5.1B,C (donor 27), induced no CMV-specific T cell proliferation. However, in a further assay with cells from different cultures from donor 2, CD40L-matured MoDC induced significant CMV-specific T cell proliferation (Figure 5.1D). The results from these proliferation assays suggested that *in vitro* induction of CMV-specific T cell proliferation was possible under certain conditions.

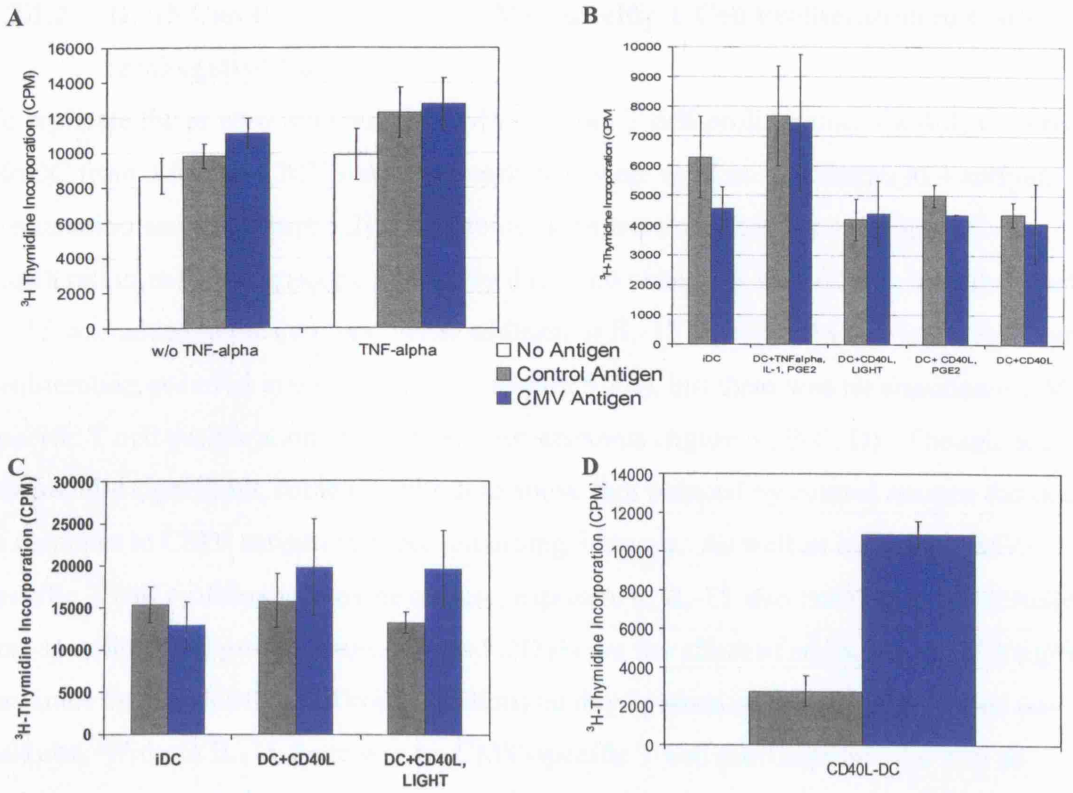

Figure 5.1 CMV Seronegative Donors - T Cell Proliferation/ Antigen Presentation Assays: Effect of DC Maturation with various Mediators.

Adherent Mo or CD14+ PBMC isolated by MACS from CMV seronegative donors were allowed to differentiate into MoDC in the presence of GM-CSF (100ng/ml) and IL-4 (100ng/ml) for 7 days. On day 4 the differentiating MoDC were pulsed with antigen, and treated with mediators of DC maturation where indicated. TNF-α (100ng/ml), CD40L (300ng/ml), and LIGHT were added to differentiating DC on day 4 (at least 3h after antigen), IL-1 (10ng/ml), and PGE_2 were added on day 6. On day 7, DC were washed and irradiated. Co-cultures were set up in triplicate wells of 96-well plates with 10 000 DC/well and between 200 000 and 300 000 autologous lymphocytes/well. Cultures were harvested between days 6 and 8, after addition of 1μCi ^{3}H-Thymidine for the final 16h before harvest. Open bars, DC pulsed with no antigen; grey bars DC pulsed with control antigen; blue bars, DC pulsed with CMV antigen; error bars, standard deviation. **A,** Donor 2, 10 000 DC + 300 000 PBL/well. Harvested on day 7; **B,** Donor 2, 10 000 DC +200 000 PBL/well, harvested on day 8; **C,** Donor 27, 10000 DC +300 000 PBL/well. Harvested on day 7; **D,** Donor 2, 5000 DC + 200 000 PBL, harvested on day 6.

5.2.1.2 IL-15 Can Increase *in vitro* CMV-Specific T Cell Proliferation in CMV Seronegative Donors

To replicate the *in vitro* induction of CMV-specific T cell proliferation, CD40L-matured MoDC from 3 further CMV seronegative donors were used as stimulators in 4 antigen-presentation assays (Figure 5.2). The co-cultures were checked for any signs of proliferation in the microscope. When by day 7, no change in the cultures was observed, IL-15 was added to the cultures. After addition of IL-15 significant CMV-specific T cell proliferation occurred in one experiment (figure 5.2A), but there was no significant CMV-specific T cell proliferation in a further 3 experiments (figure 5.2B,C, D). Though not statistically significant, some proliferation above that induced by control antigen did occur in response to CMV antigen in these remaining 3 assays. As well as inducing CMV-specific T cell proliferation in one culture, exposure to IL-15 also resulted in an increase in non-specific T cell proliferation. Figure 5.2D shows the effect of adding IL-15 (100ng/ml, ten times the previously used concentration) on day 8, when compared to untreated co-cultures. Without IL-15 there was no CMV-specific T cell proliferation. As well as slightly raising CMV-specific T cell proliferation (non-significant) IL-15 also resulted in overall non-specific T cell proliferation.

For the purpose of separating antigen-induced effects of IL-15 on T cells from any possible non-specific effects, autologous DC- T cell co-cultures were exposed to varying concentrations of IL-15. At 10 ng/ml, the dose applied in most previous experiments, IL-15 induced some non-specific T cell proliferation (Figure 5.3A), whereas at doses between 0 and 1 ng/ml no significant proliferation above the no antigen background occurred at all in two experiments (Figure 5.3A, C). In a further experiment (figure 5.3B), testing IL-15 at concentrations of up to 3ng/ml significant CMV-specific T cell proliferation only occurred in the absence of IL-15, whereas in the presence of IL-15 the difference between non-specific and CMV-specific T cell proliferation became non-significant.

IL-15 may aid the survival of activated, CMV-specific T cells. In the cultures that failed to proliferate (figure 5.3A and C) no such activation was observed using light microscopy. IL-15 may only exert an effect on CMV-specific T cell proliferation after a degree of initial antigen-induced T cell activation.

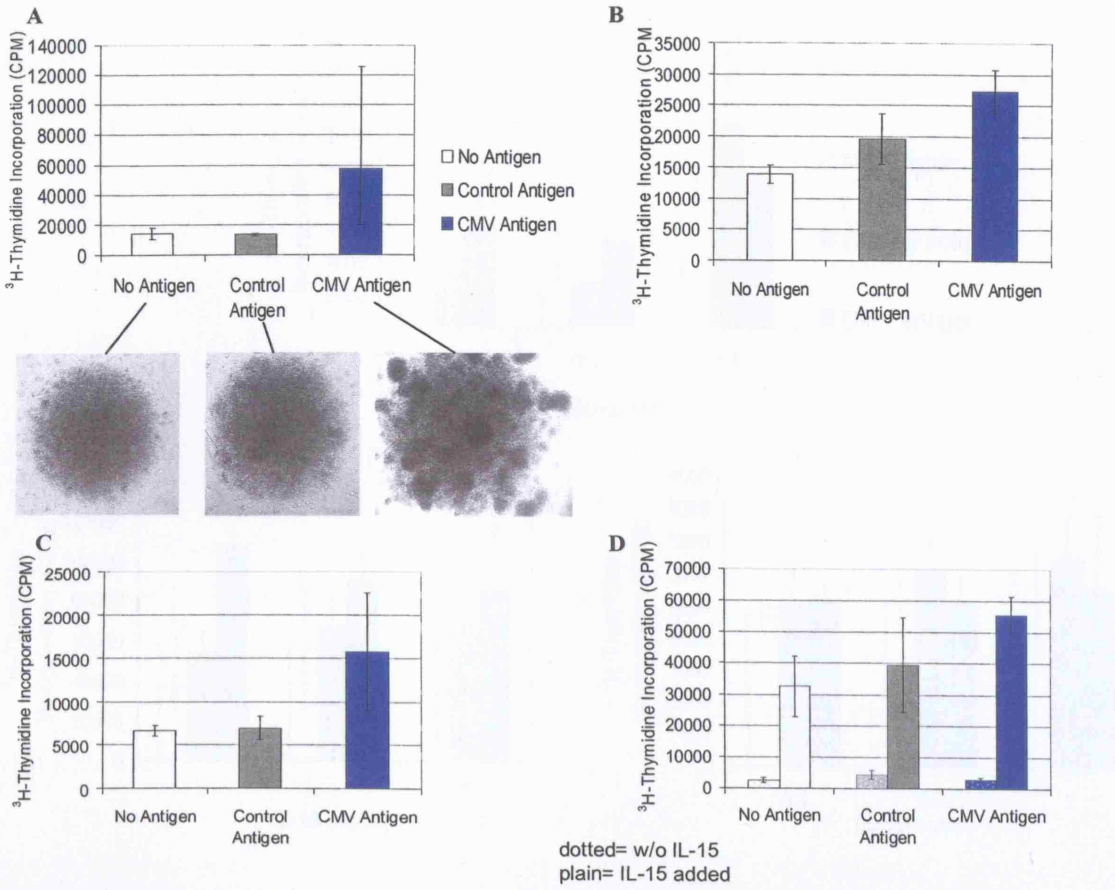

Figure 5.2 Antigen Presentation/T Cell Proliferation Assays- Effect of IL-15 in CMV Antigen-Driven Co-Cultures from CMV Seronegative Donors.

CD14-selected Mo from 3 CMV seronegative donors were differentiated into MoDC in the presence of GM-CSF and IL-4 for 7 days. On day 4, differentiating MoDC were pulsed with CMV-, control-, or no antigen, and matured with CD40L (300ng/ml) at least 3h later. On day 7, cytokines and antigens were washed off, and MoDC were co-cultured with autologous PBL in triplicate wells of 96-well plates. 16 hours before harvest, 1μCi 3H-Thymidine was added to each well. Cells were harvested onto filtermats and analysed by scintillation counting. Diagrams show mean CPM of triplicates; open bars, no antigen; grey bars, control antigen; blue bars, CMV antigen; dotted bars, no IL-15 added; error bars: standard deviation of triplicate mean, except in A, where bars show median CPM, error bars: range. **A**, CMV seronegative donor 30: Thymidine incorporation (day 10) and micrographs (day 9, x20magnification): co-cultures of 10 000 MoDC+ 150 000 PBL/well, IL-15 (10ng/ml) added to co-cultures on day 7. Cultures were harvested on day 10; **B**, CMV seronegative donor 11: co-cultures of 10 000 MoDC+ 200 000 PBL/well, IL-15 (10ng/ml) was added to co-cultures on day 7. Cultures were harvested on day 10; **C**, CMV seronegative donor 31: co-cultures of 6000 DC+ 200 000 PBL/well, IL-15 (100ng/ml) was added to co-cultures on day 7. Cultures were harvested on day **D**, CMV seronegative donor 11: co-cultures of 10 000 MoDC+ 200 000 PBL/well, IL-15 (100ng/ml) was added to co-cultures on day 7. Cultures were harvested on day 11.

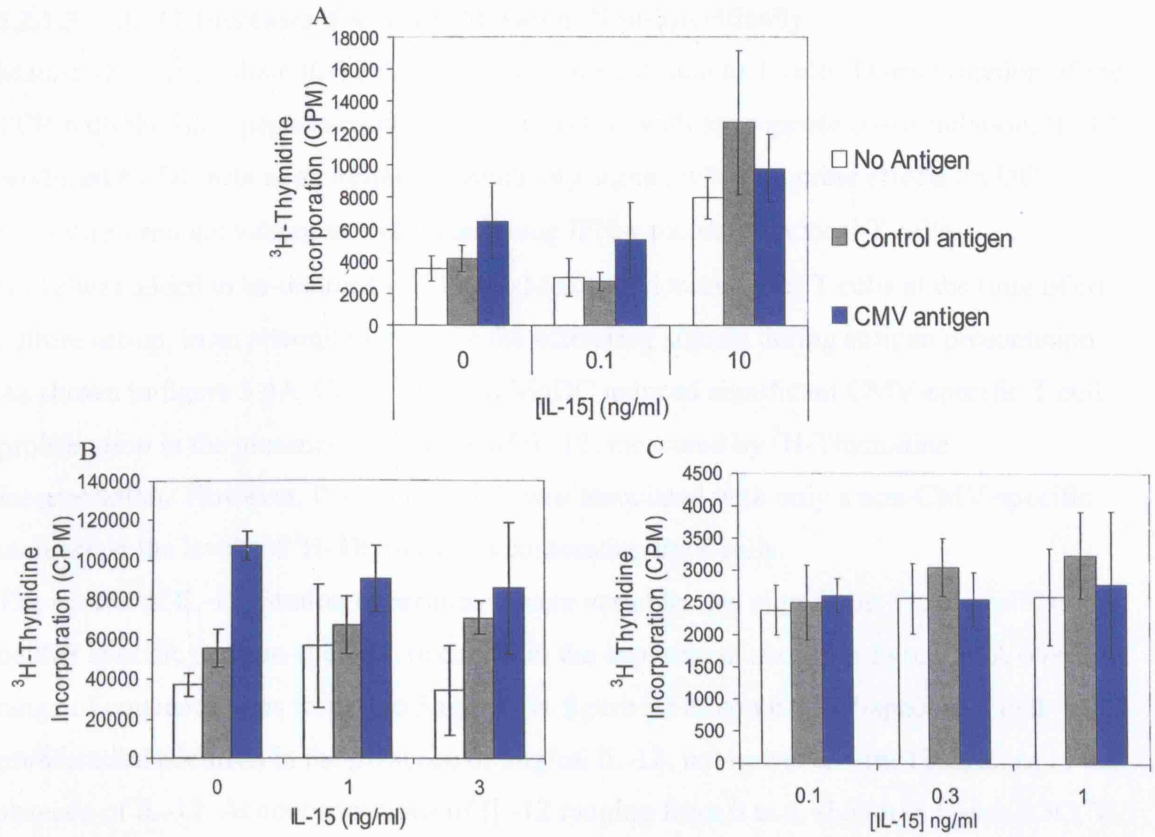

Figure 5.3 T Cell Proliferation/Antigen Presentation Assays - Titration of IL-15 Concentration.

CD14-selected Mo from 3 CMV seronegative donors were differentiated into MoDC in the presence of GM-CSF and IL-4 for 7 days. On day 4, differentiating MoDC were pulsed with CMV-, control-, or no antigen, and matured with CD40L (300ng/ml) at least 3h later. On day 7, cytokines and antigens were washed off, and 10000 MoDC were co-cultured with 150 000 autologous PBL in triplicate wells of 96-well plates. IL-15 at various concentrations was added on day 5. 16 hours before harvest, 1μCi ^{3}H-Thymidine was added to each well. Cells were harvested onto filtermats and analysed by scintillation counting. Diagrams show mean CPM of triplicates; open bars, no antigen; grey bars, control antigen; blue bars, CMV antigen; dotted bars, no IL-15 added.
A, donor 28, harvested on day 8; **B,** donor 31, harvested on day 7; **C,** donor 2, harvested on day 8.

5.2.1.3 IL-12 Increases T Cell Proliferation Non-Specifically

Mature DC can produce IL-12 during antigen presentation to T cells. During ligation of the TCR with the HLA peptide complex in conjunction with appropriate co-stimulation, IL-12 produced by DC acts as an immune-stimulatory signal, with autocrine effects on DC maturation and activation, as well as inducing IFN-γ secretion in local T cells.

IL-12 was added to co-cultures of CD40L-MoDC and autologous T cells at the time of co-culture set-up, in an attempt to enhance the activating signals during antigen presentation. As shown in figure 5.4A, CD40L-treated MoDC induced significant CMV-specific T cell proliferation in the presence or absence of IL-12, measured by ^{3}H-Thymidine incorporation. However, IL-12 (10ng/ml) was associated with only a non-CMV-specific increase in the levels of ^{3}H-Thymidine incorporation by T cells.

The results of IL-12 titration experiments were variable. No significant T cell proliferation, neither specific nor non-specific, occurred in the experiment shown in figure 5.5A over a range of concentrations from 0 to 3ng/ml. In figure 5.5B, some CMV-specific T cell proliferation occurred in the presence of 3ng/ml IL-12, not however with 10ng/ml or in the absence of IL-12. At concentrations of IL-12 ranging from 0 to 3, shown in figure 5.5C, T cells proliferated in response to CMV antigen at the middle concentration of 1ng/ml Il-12, but not at the highest concentration of 3ng/ml, or at the lowest concentration of 0.1ng/ml. Non-specific T cell proliferation in response to control antigen increased with increasing IL-12 concentration, so that at the highest concentration of 3ng/ml there was no difference in T cell proliferation induced by control antigen and CMV antigen. This would suggest that IL-12 concentrations below 3ng may enhance antigen-specific T cell proliferation.

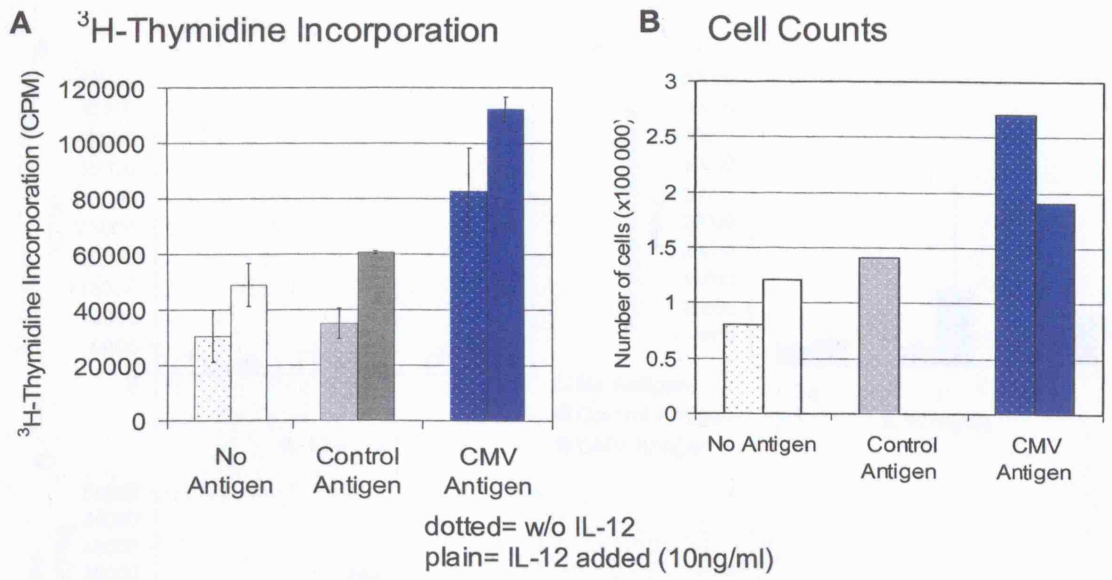

Figure 5.4 CMV Seronegative Donors: T Cell Proliferation – Effect of IL-12.

MoDC adherence-selected Mo from CMV seronegative donor 29 were differentiated into MoDC for 7 days. On day 4, differentiating MoDC were pulsed with CMV antigen, control antigen, or no antigen, and treated with CD40L (300ng/ml) 3h later. On day 7, cytokines and antigen were washed off, and MoDC were irradiated. Co-cultures with autologous T cells were set up in quadruplicate wells of 96-well plates, with 15000 MoDC and 150 000 PBL/well. IL-12 (10ng/ml) was added were indicated. Triplicate wells were harvested onto a filtermat and analysed by scintillation counting on day 7 of co-culture, with 3H-Thymidine (1μCi/well) present for the final 16h. Additionally, cells in one well from each quadruplicate were counted (except control antigen). **A,** Incorporation of ^{3}H-Thymidine; **B,** Cell count (Trypan blue excluding cells); **error bars,** standard deviation; **speckled bars**, no IL-12; **closed bars,** IL-12 (10ng/ml).

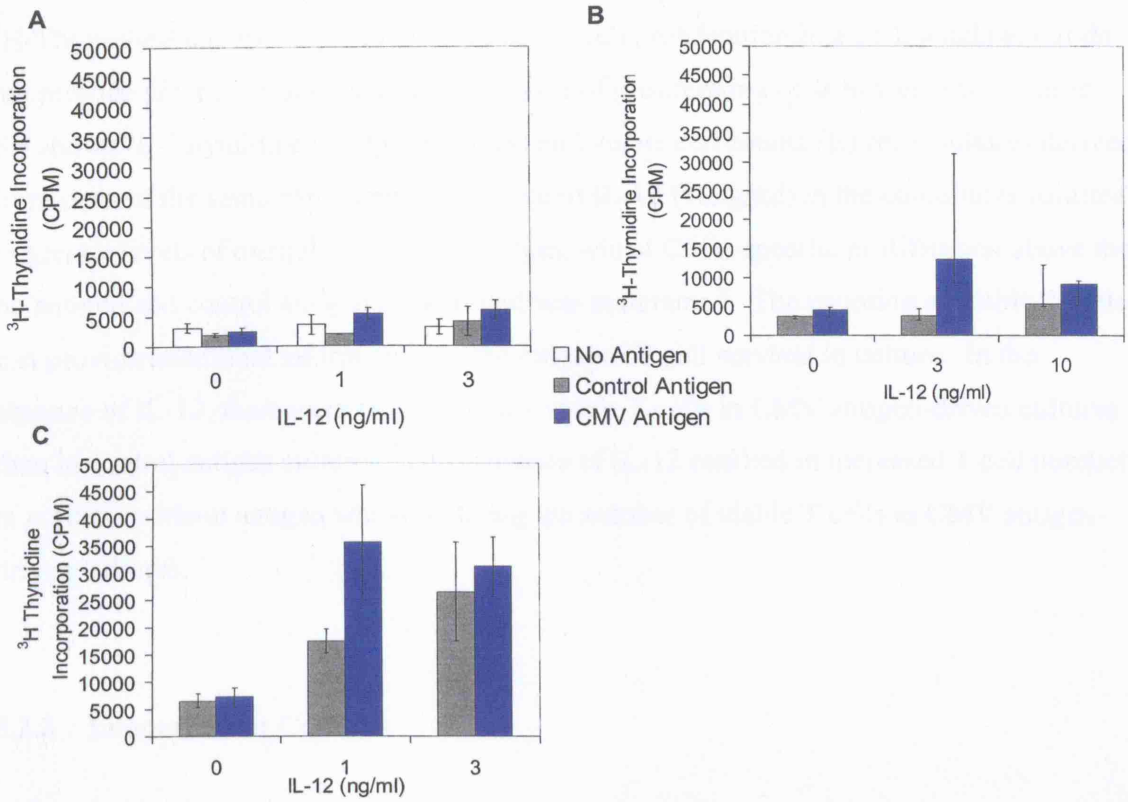

Figure 5.5 T Cell Proliferation/Antigen Presentation Assays – IL-12 Titration.

CD14-selected Mo from 3 CMV seronegative donors were differentiated into MoDC in the presence of GM-CSF and IL-4 for 7 days. On day 4, differentiating MoDC were pulsed with CMV-, control-, or no antigen, and matured with CD40L (300ng/ml) at least 3h later. On day 7, cytokines and antigens were washed off, and 10000 MoDC were co-cultured with 150 000 – 200 000 autologous PBL in triplicate wells of 96-well plates, in the presence of various concentrations of IL-12. 16 hours before harvest, 1μCi ^{3}H-Thymidine was added to each well. Cells were harvested onto filtermats and analysed by scintillation counting. Bar charts show CPM of triplicates; error bars, standard deviation of the mean. Except **B**, where bar chart shows median CPM; error bars, range. Open bars, no antigen; grey bars, control antigen; blue bars, CMV antigen. **A**, CMV seronegative donor 3, **B** and **C**, CMV seronegative donor 2.

5.2.1.4 Thymidine Incorporation Compared to Cell Counts

^{3}H-Thymidine-incorporation assays measure T cell proliferation in a 16-h window, but do not provide any information about the survival of proliferating cells beyond this. Figure 5.4 shows ^{3}H-Thymidine incorporation (A) and viable cell counts (B) from cultures derived from cells of the same blood draw. Presence of IL-12 (10ng/ml) in the co-cultures resulted in greater levels of overall T cell proliferation, whilst CMV-specific proliferation above the no antigen and control antigen background was maintained. The counting of viable T cells can provide additional information on the extent of T cell survival in culture. In the absence of IL-12, there were twice as many viable T cells in CMV antigen-driven cultures than in control antigen cultures. The presence of IL-12 resulted in increased T cell numbers in cultures without antigen whilst reducing the number of viable T cells in CMV antigen-driven cultures.

5.2.2 Longer-Term Cultures

5.2.2.1 Rationale

The ^{3}H Thymidine incorporation assays demonstrated significant CMV-specific T cell proliferation in 6/14 antigen presentation assays from 5 different donors, using CD40L-matured MoDC as APC.

Induction of primary CMV-specific T cell proliferation *in vitro* requires lasting interaction of the TCR with HLA-peptide on DC in the context sufficient levels of ligation of accessory molecules in the presence of cytokines. Induction of naïve T cell proliferation requires TCR-MHCpeptide ligation with strong co-stimulation over a longer time span than re-activation of memory T cell responses in cells from CMV positive donors. ^{3}H-Thymidine assays are useful only for measuring proliferation within a few days after culture set-up. Proliferating T cells can apoptose, resulting in a decrease in total cell number after culture, example in figure 5.4, comparing T cell proliferation to cell counts. Re-stimulation with more antigen-pulsed DC may be required for sustained T cell proliferation from CMV seronegative donors. The small media volume in the ^{3}H-Thymidine assay set-up makes re-stimulation with more antigen-pulsed MoDC impractical.

In larger cultures several rounds of re-stimulation with more antigen-pulsed MoDC can be applied. CMV-specific T cell proliferation can be evaluated by viable cell counts at different time-points, TCR CDR3 spectratyping of pre- and post-culture T cells to detect any oligoclonal T cell expansions, IFN-γ-secretion in response to re-stimulation with CMV antigen, and $HLA_{CMVpeptide}$-tetramer staining for direct labelling of CD8+ T cells with specificity for individual epitopes of CMV antigens.

5.2.2.2 *In vitro* CMV-Specific Naïve T Cell Proliferation in the Presence of IL-12 and IL-15

Larger-scale co-cultures from two CMV seronegative donors of CMV or control antigen-pulsed DC with autologous PBL were set up with and without IL-12. Development of the viable cell count over two weeks for cultures from two different donors is shown in figure 5.6. IL-15 was also added on day 8, one day after re-stimulation with more CMV antigen-pulsed DC.

As previously shown by with Thymidine incorporation assays, IL-12 can be associated with non-specific T cell proliferation (figures 5.5C and 5.4A). For this reason the concentration of IL-12 was reduced to 3ng/ml in a larger scale CMV-stimulated culture from donor 28 (Figure 5.6A and table 5.1, donor 28) and compared to an identical culture set up in parallel w/o IL-12. The change in cell number throughout 14-day culture is shown in figure 5.6A. With or without IL-12, there was a reduction in cell number in the first week of culture, which was steeper in the presence of IL-12. In the second week of culture after re-seeding and restimulating with fresh DC, and addition of IL-15, T cell numbers recovered again to greater than pre-culture levels in the absence of IL-12, and to below initial levels in the presence of IL-12. This suggested that even when combined with IL-15, IL-12 can result in lower T cell numbers.

Using cells from another donor (donor 11, table 5.1 and figure 5.6B), cultures containing cytokines (IL-12 and IL-15) were compared to cultures without added cytokines, and control antigen cultures were set up alongside CMV antigen cultures to test antigen-

specificity. The development of T cell counts over time is shown in figure 5.6B. In the cultures without IL-12, a slight increase in cell counts in the presence of CMV antigen-pulsed DC, but not control antigen-pulsed DC indicated some CMV-specific T cell proliferation by day 7. In the presence of IL-12, proliferation occurred to the same degree with both control- and CMV antigen, indicating some non-CMV-specific T cell proliferation. Cultures were re-plated and re-stimulated with more CMV-antigen-pulsed MoDC at a ratio of 20:1 on day 7. Additionally, the IL-12-containing cultures were exposed to IL-15 one day after re-stimulation with DC. On day 14 the cultures were harvested and counted. In all but one culture T cell counts had decreased by day 14. Only in the culture with CMV antigen and in the presence of the cytokines there was an increase in cell number between days 7 and 14 of culture. In this culture the number of viable T cells had more than doubled during the final week of culture. Furthermore, only in the CMV/cytokine culture did T cells appear activated in the light microscope, i.e. in contact with DC, teardrop-shaped. This was evidence of sustained CMV-specific T cell proliferation in the presence of IL-12 and IL-15. The results from both experiments together would suggest expansion of CMV-specific T cells due to the presence of IL-15, but not IL-12.

To test reproducibility and to allow differentiation between the relative effects of IL-12 and IL-15, we looked at eleven 2-week cultures from 7 donors in total. T cell counts and fold expansion are displayed in table 5.1. The presence of IL-12 or IL-15 in the cultures is indicated next to the cell counts. Since availability of MoDC, which were required for initial culture set-up and any re-stimulations, was always a limiting factor, most cultures were set up with CMV antigen-pulsed DC only, without control antigen and no antigen control cultures.

In 8/12 cultures T cell proliferation occurred during the first week of culture, and in 8/12 cultures T cells proliferated in the second week of culture. Two cultures proliferated in the first week with no further proliferation in the second week; three proliferated in the second week only. In 5/12 cultures there was T cell proliferation in both weeks. One culture failed to proliferate throughout. Cultures from donor 29 were set up with and without IL-15, added from day 8 onwards, showing greater T cell proliferation in the presence of IL-15.

The cultures from donor 2, set up with and without IL-12 from day 0, indicate that IL-12 can initially lead to more rapid proliferation, which is followed by greater cell death. T cell counts from donors 29 and 2 show that IL-12 alone does not result in a greater yield of post-culture T cells, but IL-15 on its own does.

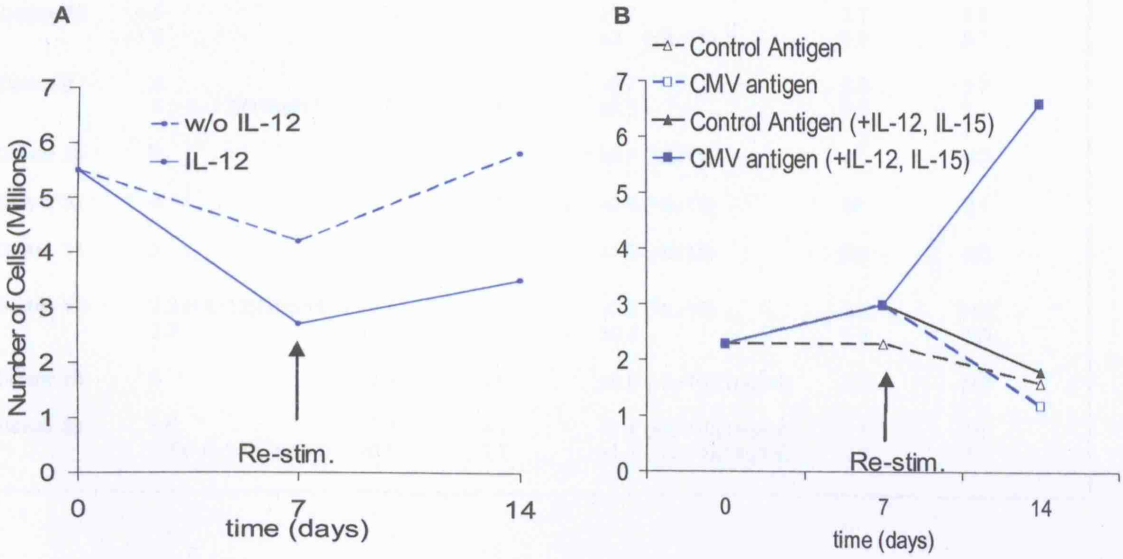

Figure 5.6 CMV Seronegative Donors: 2-Week Cultures – Cell Counts.

Monocytes were differentiated into DC over 7 days. Differentiating MoDC were pulsed with CMV or control antigen on day 4, and matured with CD40L (300ng/ml) 3h later. On day 7, cytokines and antigens were washed off, and MoDC were co-cultured with autologous PBL/well with or without IL-12 (10ng/ml). Co-cultures were re-stimulated with more antigen-pulsed DC on day 7. Co-cultures were maintained for 14 days. Graphs show counts of Trypan blue-excluding cells. **A,** Donor 28 culture, DC were derived from adherence-selected Mo and pulsed with CMV antigen. Co-cultures with or without IL-12 (3ng/ml) from day 0. IL-15 (1ng/ml) was added to both cultures on day 11. **B,** Donor 11, DC were derived from CD14-selected Mo pulsed with CMV or control antigen. IL-12 (10ng/ml) was added to co-cultures on day 0, where indicated. IL-15 was added on day 8, one day after re-stimulation, where indicated.

CMV Seronegative Donors: Longer-Term Cultures

A) Culture Conditions

Day 0 →	1 week →	1 week+1 day →	2 weeks
Co-cultures (+/-IL-12)	restimulate with CMV-DC	(+/-IL-15)	harvest

B) Cell number, fold expansion, ratio of CD4:8 expression of post-culture T cells

CMVneg donor	Day 0 count (millions)	fold expansion	week 1 count	fold expansion	week 2 count	CD4/ CD8ratio
Donor 29	1.5	x1.4	2.2	x1.7	3.7	8.6
	1.5	x1.4	2.2	x3 (+IL-15)	6.6	8.7
Donor 2	4	x2	8	x0.7	5.6	5.7
	4 (+IL-12)(10ng/ml)	x2.8	11	x0.5	5.6	2
Donor 30	2	x1.5	3	x5.7 (+IL-15)	17	ND
Donor 32	8	x4.3	34	x2.6 (+IL-15)	88	2.1
Donor 11	5	x1	5.2	x1.3 (+IL-15)	6.8	ND
Donor 11	2.3 (+IL-12)(3ng/ml)	x1.3	3	x2.2 (+IL-15)	6.6	ND
	2.3	x1.3	3	x0.4	1.2	ND
Donor 33	5	x0.8	3.8	x0.6 (+IL-15)(1ng/ml)	2.2	ND
Donor 28	5.5	x0.8	4.2	x1.4 (+IL-15)(1ng/ml)	5.8	10
	5.5 (+IL-12)(3ng/ml)	x0.5	2.7	x1.3 (+IL-15)(1ng/ml)	3.5	7

Table 5.1 CMV Seronegative Donors – Longer-Term Cultures.

Co-cultures of CMV antigen-pulsed MoDC and autologous PBL were set up on day 0. Initial numbers of PBL, fold-expansion, and weekly cell counts are displayed in the columns. The addition of cytokines, IL-12, and IL-15 (3ng/ml) is indicated in brackets. The r.h.s. column shows post-culture CD4:CD8 ratios.

5.2.2.3 Immunophenotype of the Culture Output from CMV Seronegative Donors

Post-culture ratios of CD4 and CD8 expressing T cells from four different CMV seronegative donors are displayed in table 5.1. In the culture output from 3/4 donors, the ratio indicated greater proliferation in the CD4 T cell compartment, whereas in one donor (donor 32) the ratio was unchanged from pre-culture, suggesting equal proliferation in CD4 and CD8 T cell compartments. The presence of IL-12 in the cultures affected CD4:CD8 ratios. There was no change in the CD4:CD8 ratio from pre- to post-culture in the presence of IL-12 in cultures from donor 2, but without IL-12 cultures were skewed towards CD4. In both of these cultures cell number only increased in the first week, with a reduction in cell number by the end of the second week of culture. The drop in cell number was steeper in IL-12 cultures than in cultures not exposed to IL-12. The lower frequency of post-culture CD4+ T cells in IL-12 cultures could thus be a result of greater CD4 T cell death, or lower CD4+ T cell proliferation compared to cultures without IL-12.

IL-15, on the other hand, had no further effect on post-culture CD4:CD8 ratios. For donor 29 there were twice as many post-culture T cells in the presence of IL-15 than without IL-15 by the end of two weeks, but CD4:CD8 ratios were the same with- or without IL-15. T cells in both cultures were skewed towards CD4 expression to the same degree compared to pre-culture. Cell number had increased in these cultures in the first and second week of culture, more so after addition of IL-15 on day 8. IL-15 appears to serve as a growth stimulus for both CD4 and CD8 T cells.

5.2.2.4 Post-Culture TCR CDR3 Spectratypes Indicate Oligoclonal T cell Expansion Induced by CMV Antigen-Pulsed DC in CMV Seronegative Donor Cultures

Comparative TCR CDR3 spectratyping can serve to detect oligoclonal T cell expansions, for example in response to stimulation with a specific antigen, such as CMV. A marked change in TCR CDR3 spectratype patterns from pre- to post- culture in conjunction with an increase in cell number would indicate expansion of certain T cell clones, induced by exposure to antigen. On the other hand, if T cell proliferation were non-antigen specific, there would be an increase in T cell number, but TCR CDR3 spectratype patterns would not be expected to change markedly compared to uncultured T cells.

Many of the pre-culture spectratype histograms from donor 11 displayed in figure 5.7 show the typical near-normal distribution of PCR product amongst the different size classes indicative of un-stimulated T cells. Pre-culture spectratypes were nearly identical to post-culture spectratypes from 2-week cultures induced by control antigen-pulsed DC, indicating non-detectable levels of T cell expansion in response to control antigen. However, T cells induced with CMV antigen for two weeks gave rise to some very skewed spectratypes in at least 9/23 BV families, when compared to those from the control antigen-induced cultures grown from the same blood draw. This strongly suggested expansion of T cell clones in response to CMV antigen.

Similarly, TCR CDR3 spectratyping of pre- and post culture T cells from donor 29 provided further evidence for CMV-specific T cell expansion. Examples of pre- and post-culture spectratypes for two BV families are displayed in Figure 5.8. After 2-week co-culture with CMV antigen-pulsed MoDC, T cells had expanded 4-fold in number (see table 5.1), and gave rise to very skewed TCR spectratypes, with some very prominent peaks. Post-culture spectratypes for the same BV-families were very reproducible; there was very little difference between TCR spectratypes from CMV antigen-induced cultures with or without IL-15. The only difference was that T cell spectratypes from cultures with IL-15 tended to be skewed to an even greater extent than those from cultures without IL-15, but with a very similar basic shape.

The great similarity between spectratypes from separate cultures from the same donor shows that the same oligoclonal T cell expansions were reproducibly induced in response to CMV antigen. Even though IL-15 has been shown to induce some overall T cell proliferation, as shown by ^{3}H-Thymidine incorporation assays (Figure 5.3A), it can exert a greater effect on T cells pre-activated by exposure to antigen, giving rise to very skewed post-culture spectratypes.

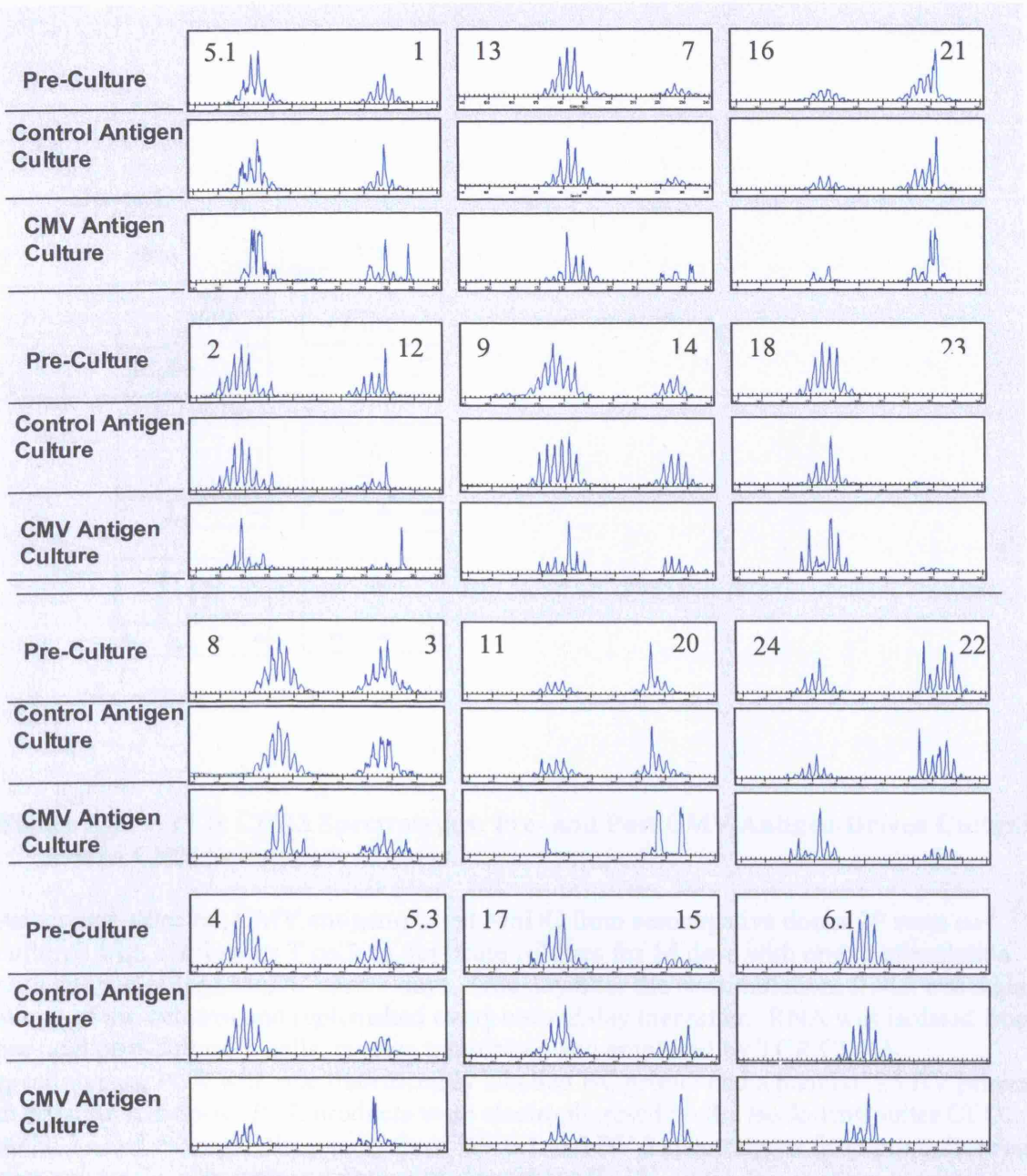

Figure 5.7 Pre-Culture and Post-Culture Spectratypes: CMV Seronegative Donor
CMV- or control antigen-pulsed MoDC generated from magnetic activated sorted CD14+ PBMC from CMV seronegative donor 11 were co-cultured with autologous PBL in the presence of IL-12 (100 pg/ml). Cultures were restimulated with antigen-pulsed MoDC on day 7. IL-15 (10ng/ml) was added to the co-cultures on day 9. RNA extracted from pre- and post-culture cells was reverse transcribed and amplified by TCR CDR3 spectratyping PCR with one fluorescently labelled BC primer and a panel of 23 BV primers in separate reactions. PCR products were electrophoresed on the BeckmanCoulter CEQ 8000. The BV subfamily corresponding to each spectratype is indicated by a number above.

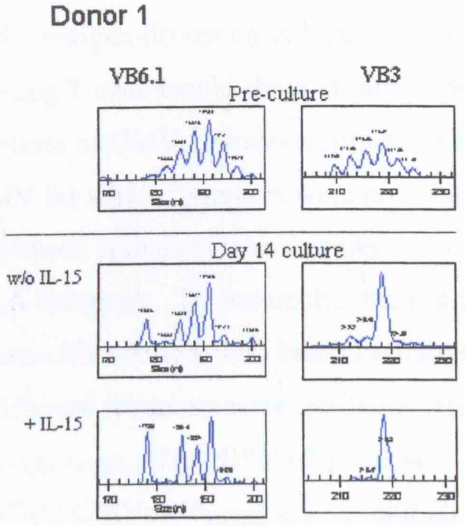

Figure 5.8 TCR CDR3 Spectratypes: Pre- and Post CMV Antigen-Driven Culture from a CMV Seronegative Donor.

Adherence-selected, CMV antigen-pulsed MoDC from seronegative donor 29 were co-cultured with autologous T cells in duplicate cultures for 14 days with one re-stimulation with antigen-pulsed MoDC after 7 days. One day after the re-stimulation, IL-15 was added to one of the cultures and replenished every second day thereafter. RNA was isolated from pre- and post-culture T cells, reverse transcribed and amplified by TCR CDR3 spectratyping PCR with one fluorescently labelled BC primer and a panel of 23 BV primers in separate reactions. PCR products were electrophoresed on the BeckmanCoulter CEQ 8000. As and example, the panels show BV 6.1 and BV 3 spectratypes from pre-culture and post-culture T cells, from cultures with- or without IL-15.

5.2.2.5 HLA-CMVpeptide-Tetramer Staining of Post-Culture T cells from CMV Seronegative Donors

The detection of HLA-CMVpeptide-tetramer-binding T cells in the culture output would provide ultimate proof that naïve precursor CMV-specific T proliferation had occurred in CMV antigen-driven co-cultures from CMV seronegative donors. Lack of tetramer-binding T cells would show failure of proliferation of T cells specific for those individual peptides of CMV. However, proliferation of CD8+ T cells specific for other peptides of CMV for which tetramers were not available could still have occurred. Culture output from two-week antigen-driven cultures from four CMV seronegative donors was stained with HLA tetramers. To ensure that the staining worked, the culture output from CMV seropositive donors with known tetramer positivity was stained at the same time. A total of 6 different tetramers were available: HLA A*A0201-CMVpp65$_{NLVPMVATV}$, HLA A*0201-IE1$_{VLEETSVML}$, HLA B*0702 pp65$_{TPRVTGGGAM}$, HLA A*2402-CMVpp65$_{QYDPVAALF}$, HLA B*0702-CMVpp65$_{RPHERNGFTVL}$, and HLA A*2402-CMVpp65$_{VYALPLKML}$. The culture output from each donor was stained with at least two and up to 3 (donor 2) different tetrameric complexes, each staining T cells specific for a single peptide of CMV, restricted by a specific type of HLA class I. An example from one CMV positive and one CMV negative donor is shown in Figure 5.9. Tetramer-binding T cells could not be detected in any of the culture outputs from all four CMV seronegative donors. However, due to high background staining the specificity of staining was low, and it would not be possible to distinguish very low frequencies of tetramer-binding cells from the background.

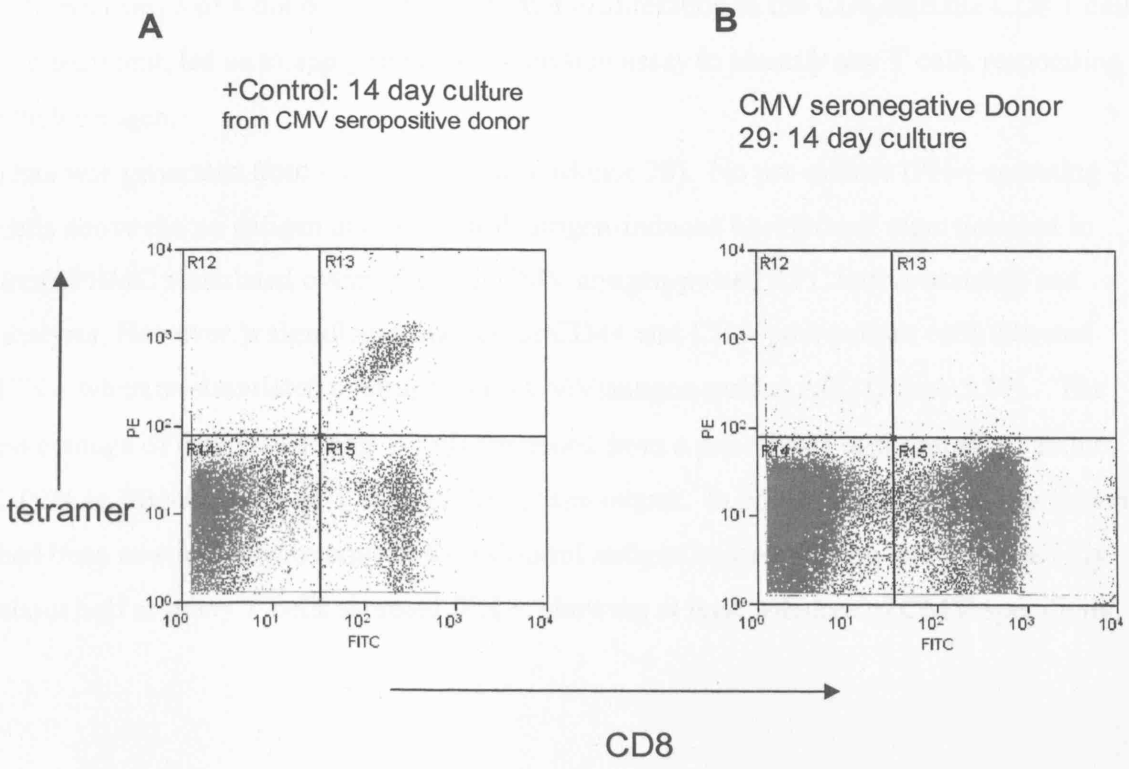

Figure 5.9 CMV Seronegative Cultures – HLA-CMVpeptide-Tetramer Staining

Flow diagrams of cultured T cells from HLA A*0201-expressing donors stained with HLA A*0201 pp65NLVPMVATV--tetramer
A, Positive control: 14-day CMV antigen-driven culture from a CMV seropositive donor.
B, 14-day CMV antigen-driven culture from CMV seronegative donor 29.

5.2.2.6 IFN-γ Secretion by T Cells from CMV Seronegative Donors: Pre-Culture and Post CMV Antigen–Driven Culture

Cytokine secretion assays can be used to detect T cells responding to any antigen of choice. The failure to detect HLA CMVpeptide tetramer-binding T cells, and the fact that in cultures from 3 of 4 donors there was greater proliferation in the CD4 than the CD8 T cell compartment, led us to apply the IFN-γ secretion assay to identify any T cells responding to CMV antigen.

Data was generated from one donor culture (donor 28). No pre-culture IFN-γ-secreting T cells above the no antigen and/or control antigen-induced background were detected in fresh PBMC stimulated overnight with CMV antigen-pulsed APC before staining and analysis. However, a significant fraction of CD4+ and CD4- post-culture cells secreted IFN-γ when re-stimulated overnight with CMV antigen-pulsed APC (figure 5.10). The percentage of IFN-γ-secreting T cells increased from a pre-culture background of about 0.02% in PBMC to 1% of T cells in the culture output. In post-culture populations which had been re-stimulated overnight with Control antigen before staining and analysis only about half as many T cells secreted IFN-γ, showing at least a degree of CMV-specificity.

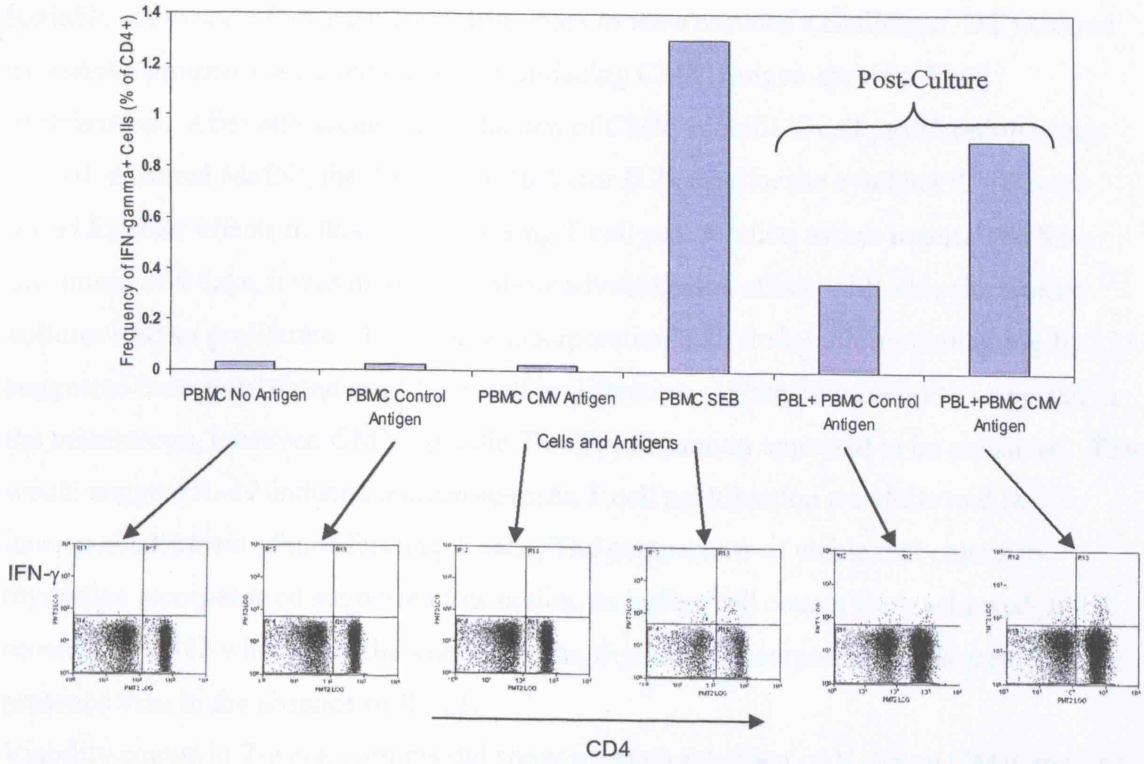

Figure 5.10 IFN-γ-Secretion by T Cells from a CMV Seronegative Donor after CMV Antigen-Driven Culture.

Adherence-selected Mo from CMV seronegative donor 28 were allowed to differentiate into DC in the presence of GM-CSF and IL-4 for 6 days. Differentiating MoDC were pulsed with CMV antigen on day 4 and matured with CD40L 3h later. MoDC were co-cultured with autologous lymphocytes for 14 days, with one further re-stimulation with antigen-pulsed DC on day 7. IL-15 (1ng/ml) was added from day 8. Post-culture PBL were frozen. For the IFN-γ-secretion assay, post culture cells were thawed and restimulated o/n with Control antigen or CMV antigen-pulsed fresh PBMC at a ratio of 4:1. Fresh PBMC pulsed overnight with no antigen, control antigen, CMV antigen, or SEB served as controls.

A, % of CD4+ T cells secreting IFN-γ; B, flow diagrams: cells were labelled with CD4-FITC, IFN- γ-PE, dead cells were excluded by gating on the PI-excluding cell fraction.

5.3 Discussion

Reliable activation of primary T cell responses *in vitro* remains a challenge. DC matured by various protocols were not capable of inducing CMV antigen-specific T cell proliferation. After one successful induction of CMV-specific T cell proliferation using CD40L-matured MoDC, the T cell growth factor IL-15 and/or the cytokine IL-12 were tested for their effects in this system. Using T cell proliferation assays maintained for a maximum of 9 days, it was difficult to show advantageous effects of IL-15, since many cultures did not proliferate. Thymidine incorporation data from cultures containing IL-12 suggested induction of non-specific T cell proliferation. When cultures were inspected in the microscope, however, CMV-specific T cell proliferation appeared to be enhanced. This would suggest IL-12-induced antigen-specific T cell proliferation was followed by increased apoptosis of proliferating T cells. The comparison of viable cell counts to thymidine incorporation supported this notion, as higher cell counts were achieved in the absence of IL-12 whereas in the same cultures, thymidine incorporation was greater in the presence than in the absence of IL-12.

Viability counts in 2-week cultures did show a beneficial effect of IL-15 on CMV-specific T cell proliferation. However, the amount of non-specific T cell proliferation induced by IL-15 will need to be more accurately determined, and timing and dose of IL-15 require careful titration.

When setting up larger scale cultures, the priority was to obtain enough cells for tetramer staining and cytokine secretion assays. For this reason control antigen cultures were not always available, and increases in the cell counts of CMV-antigen driven cultures may or may not have been CMV specific. However, strong evidence for CMV specificity of one longer-term culture was provided by TCR CDR3 spectratyping data. Spectratypes from CMV antigen-driven cultures were skewed, whereas spectratypes from Control antigen-driven cultures were nearly identical to pre-culture spectratypes.

While there was no detectable increase in HLA-tetramer-binding cells above background, the possibility of presence of a very small population of tetramer-positive T cells could not be ruled out, but the vast majority of CD8+ T cells definitely did not bind HLA-$CMV_{peptide}$-tetramers.

The γ-IFN secretion assay generated meaningful data from only one donor, identifying a post-culture T cell population responding to CMV antigen. As expected, no CMV-specific IFN-γ secreting T cells were detected in pre-culture PBMC. Levels of precursors in virus naïve individuals would be expected to be extremely low, so as to not be distinguishable from the background levels of IFN-γ secreting T cells. In addition, a 16-h stimulation with antigen would not be expected to be long enough to activate naïve T cells. After CMV antigen-driven culture, however, a population of T cells secreted IFN-γ after re-stimulation with CMV or control antigen. As expected, re-stimulation with CMV antigen induced IFN-γ secretion in a greater percentage of T cells than re-stimulation with control antigen, indicating a degree of CMV-specificity of the culture output. However, the presence of CMV-antigen-presenting DC in the culture output, hindered the quantification of CMV-specific versus control antigen-specific responses.

CHAPTER 6

Conclusions

MoDC play a key role as APC in the CMV antigen-driven T cell culture system. Chapter 3 describes different approaches of optimising *in vitro* DC maturation, for obtaining the most effective APC. Various DC maturation protocols from the published literature were utilised and modified where found necessary. MoDC matured with cytokines, pro-inflammatory mediators, soluble T cell surface molecules, or combinations of these, were compared to untreated MoDC and to each other with respect to surface marker expression and as inducers of antigen-specific T cell proliferation. CD40L was the most promising candidate for DC maturation in this culture system, because compared to DC matured with other mediators, CD40L-DC induced the least non-CMV-specific T cell proliferation in response to control antigen. Exposure of DC to soluble CD40L resulted in only intermediate maturation marker expression, but increased capacity for induction of antigen-specific T cell proliferation. Combining CD40L with other mediators was of no further benefit.

In larger scale cultures, equivalent to those employed for the generation of T cells for adoptive immunotherapy, CD40L-DC were shown to induce antigen-specific T cell proliferation under conditions in which untreated DC did not induce T cell proliferation. Like untreated MoDC, CD40L-DC induced proliferation in both, CD4 and CD8 T cell compartments. A larger proportion of T cells induced by CD40L-DC secreted IFN-γ upon re-stimulation with CMV antigen. Because of these advantageous properties of CD40L-DC, the protocol for generation of CMV-specific T cells for immunotherapy was modified to include CD40L-mediated maturation of DC for the initial T cell induction.

CD40 ligation using soluble CD40L was shown here to be an effective way of inducing DC maturation. CD40 ligation can also be achieved using anti-CD40 antibodies (e.g.[146]). Cross-ligation of CD40 on APC may be enhanced by utilising a molecular CD40L cross-linker that is commercially available. Further work exploring these different options could identify the most effective way of achieving CD40 ligation.

During the course of several DC maturation experiments it became apparent that CD83 expression by DC did not directly relate to their functional properties as APC. Several additional ways to assess DC are available and have been employed by others. Migratory properties of DC can be assessed through transwell migration assays (e.g. [103;116]). ELISAs

[103;106;110-112;115;118;119;147;148], or real-time PCR [148;103;119] can be employed to detect cytokine expression or transcription. Antigen uptake by DC can be quantified by measuring ingestion of fluorescent-labelled dextran or beads [103;147;148]. In the future, such additional tests could provide a more complete functional profile of MoDC matured by the different methods tested here.

To optimize the system for the *in vitro* generation of CMV-specific T cells for immunotherapy further, the effects of different methods of DC antigen pulsing were investigated. This revealed variability in T cell toxicity between different batches of CMV antigen, and led to the decision to routinely remove soluble antigen before T cell culture to avoid any inhibitory effects of the antigen. Concentration of CMV antigen and the time of antigen addition during DC differentiation for induction of maximum T cell proliferation were determined.
Further optimisation of the timing of CMV antigen pulsing in the context of DC maturation may be useful. CMV antigen is routinely added to DC a minimum of 3h before addition of CD40L. But whether 3h is adequate to allow optimal uptake and processing of CMV antigen before DC maturation remains to be determined experimentally.

The output of MoDC in cultures derived from the adherent fraction of PBMC contains a host of different as yet uncharacterised cell types. Only a variable proportion of cells in these cultures display DC morphology and surface marker expression. Analysing adherent and non-adherent fractions separately showed that non- and lightly adherent cells induced antigen-specific T cell proliferation, whereas adherent cells did not.
The presence of macrophages in DC cultures, for example, may have an inhibitory effect on T cell activation. A more detailed characterization of the cells resulting from culture of adherent PBMC in the presence of GM-CSF and IL-4 by immunophenotyping and the effect of the cell types on T cell activation may be of interest, not least because this could enable us to obtain a purer population of the most effective APC.

Availability of DC is often a limiting factor in the maintenance of CMV-antigen-driven T cell cultures. Using cryopreserved/thawed MoDC derived from fresh PBMC in re-

stimulations increased DC yield, when compared to MoDC generated from previously cryopreserved PBMC.

The potential exists for further work exploring the use of other cell types, e.g. monocytes for re-stimulations, or alternatively non-specific expansion of T cells with antibody-coated beads [149], chemicals such as PHA or antibodies, such as OKT3 [59;150] and/or cytokines, such al IL-2 (e.g. [58;62]) with or without feeder cells after the initial induction with antigen-pulsed MoDC can be explored.

T cells from CMV antigen driven cultures were characterized using the IFN-γ secretion assay. T cells secreting IFN-γ after short, specific re-stimulation were then subjected to further analysis by TCR CDR3 spectratyping.

The IFN-γ secretion assay, typically utilised for the isolation of antigen-specific T cells directly from peripheral blood or fresh PBMC, was modified to detect cells that had already undergone massive antigen-specific proliferation *in vitro*. The dynamics of IFN-γ secretion were explored in a time course experiment relating total T cell number to the number and percentage of IFN-γ-secreting T cells. Identification of the culture stage at which a maximum total number and proportion of T cells secrete IFN-γ may have implications for future decisions regarding the timing of culture harvest for adoptive cellular therapy. The maximum percentage of T cells secreted IFN-γ shortly after culture growth began to slow-down.

There was a significant increase in frequencies of IFN-γ secreting T cells from pre- to post-culture in 100% of cultures from 9 different donors when measured at the estimated time of maximum IFN-γ secretion.

IFN-γ-secreting T cells have been shown by others to possess cytotoxic properties [151], but to establish whether harvesting cultures at the time of maximum IFN-γ secretion would improve anti-CMV effectiveness *in vivo,* further functional assays comparing cultures harvested at different stages, or comparing different constituents of the culture output would be required.

Antigen-specific T cell populations isolated from CMV antigen driven cultures by IFN-γ secretion assay may be further expanded non-specifically using cytokines that promote T cell proliferation.

A preliminary experiment with cells from one donor showed that total number and proportion of antigen–induced IFN-γ-secreting T cells may be increased by non-specific expansion. After a 7-day non-specific expansion of the post-culture IFN-γ secreting T cell fraction, the percentage of T cells producing IFN-γ was nearly 10-fold greater than in un-selected T cells straight after antigen-driven culture. 50% of post-expansion T cells were IFN-γ-secreting when re-challenged with CMV antigen. However CD4+ T cells divided at a faster rate than CD8+ T cells under these conditions, so that the bulk of post-expansion T cells were of the CD4 phenotype, which is consistent with observations by Einsele *et al.* [52]. Expansion of IFN-γ+ T cells was enhanced in the presence of IL-7. This cytokine would also be present at high concentrations *in vivo* in the blood of patients re-constituting T cell numbers post-HSCT/BMT.

The dynamics of IFN-γ secretion across the repertoire of T cell CDR3 BV size classes at different time points was monitored through TCR CDR3 spectratyping. The spectratyping data strongly suggested in some donors IFN-γ was secreted by members of the same T cell clones from day 7 through day 13 of culture, i.e. IFN-γ secreting T cells tended to express the same set of BV size classes across 23 BV families, and spectratype shapes did not change markedly over time. This would suggest that T cells became activated by the same epitopes of CMV over time. This may be surprising considering T cells are exposed to a range of optimal and suboptimal epitopes, and could be expected to vary in their activation thresholds However, in other donors, some spectratypes were dominated by different BV size classes at different times, indicating a greater range of threshold levels for re-activation between different CMV specific T cell clones. Re-activated T cells are memory T cells and as such have very low activation thresholds. This may explain the overall similarities between BV size class expression in CMV antigen-driven cultures over time. More differences may be detected at the earlier stages of culture, and may warrant further time-course experiments.

It would be of value to establish the generality of these findings in a time course in cultures from several different donors.

The IFN-γ secretion assay can detect T cells of both CD4 and CD8 phenotype responding to antigens of interest, whereas HLA-tetramers or pentamers allow focusing on CD8+ T cells specific for single epitopes without the functional dimension.
HLA-tetramer staining was applied here to detect pre- and post-culture levels of T cells specific for immunodominant epitopes of CMV pp65 or IE-1, restricted by HLA*A2- or B7.
In 90% of cultures there was an increase in frequencies of CD8+ T cells specific for one or more of the three epitopes of CMV from pre- to post- culture. Our data also confirmed the previously observed skewing of the T cell response to pp65 toward B7-restriction in individuals expressing both HLA A2 and B7 [141].

Tetramer technology requires prior knowledge of immunodominant epitopes and their HLA-binding. As more immunodominant epitopes of CMV are identified, greater opportunities will arise for the application of tetramer staining as a tool for isolating CMV-specific T cells.
We utilised comparative TCR CDR3 spectratyping to get an impression of the contribution of T cells specific for single immunodominant epitopes, as identified by tetramer staining, and of T cells secreting IFN-γ in response to CMV lysate to the full *in vitro* T cell response. As expected, there was a degree of overlap in BV size-class expression between the post-culture IFN-γ-secreting- and tetramer-binding populations. However, not all BV size classes expressed by tetramer-binding T cell clones were also expressed by IFN-γ-secreting CD8+ T cells, indicating that some CMV-specific CD8+ T cell clones could not easily be reactivated to produce IFN-γ.
As expected, IFN-γ-secreting/CD8+ T cells expressed a greater range of BV size classes than tetramer-binding T cells, which indicated their specificity for several epitopes, possibly from a greater range of antigen constituents of the CMV lysate than those detected by the tetramers. As well as by HLA-A*0201 or HLA-B*0702, IFN-γ-selected CD8+ T cells may be restricted by other HLA alleles where available, further explaining their greater spectratype diversity.

Both, the IFN-γ assay and tetramer staining failed to detect large numbers of the T cells shown to have proliferated in response to CMV antigen. A large number of post-culture CMV-specific T cells could not be re-stimulated to produce IFN-γ, but the IFN-γ-secreting sub-population tended to cover the full range of the CMV-specific T cell repertoire, as shown by TCR CDR3 spectratyping.

Any relevance of these findings to clinical applications ultimately remains to be determined in clinical follow-up studies. Further *in vitro* tests, such as cytotoxicity assays using the different T cell subpopulations, may help identify the likely minimum requirement for a sustained protective immune response against CMV.

Combination IFN-γ-labelling and tetramer staining enables direct visualisation of individual antigen-specific T cells responding to antigen challenge, and have been combined by several authors [142;152;153] using multiplex staining protocols. But RNA for TCR CDR3 spectratyping required for the appreciation of the diversity of T cell responses, can only be extracted from unfixed cells, such as those obtained in the IFN-secretion assay [154], but not through intracellular cytokine staining protocols.

The induction of naïve CMV-specific T cells *in vitro* has so far been met with only partial success. *In vivo*, the high activation thresholds of naïve T cells, which can only be overcome under very specific conditions within the lymph nodes, help prevent damaging effects of inappropriate immune activation.

Mature DC are the only antigen presenting cells known capable of inducing primary T cell responses.

Utilising CD40L-matured MoDC, we have demonstrated CMV antigen-induced T cell proliferation *in vitro* using cells from donors who tested negative for CMV-specific antibodies and CMV DNA. Strong evidence for proliferation of CMV specific T cell clones came from TCR CDR3 spectratypes, which indicated oligoclonal expansion of T cells in response to CMV antigen, but not control antigen. However, CMV-specific CD8+ T cells specific for the immunodominant epitopes of CMV currently available as HLA-tetramers could not be detected in any of the cultures, which was perhaps not surprising considering the very low precursor frequencies of CD8+ CMV-specific T cells circulating in the blood.

In the future, monitoring cultures by a greater range of tetramers may become possible, as more of the immunodominant epitopes of CMV binding different HLA-alleles are identified.

The measurement of IFN-γ secretion was met with technical difficulties, i.e. high background staining for most CMV seronegative cultures. Hence, not much data on IFN-γ-producing T cells in cultures from CMV seronegative donors is available so far, however the results from one culture show T cells producing IFN-γ in response to CMV antigen. This particular experiment showed greater IFN-γ secretion by a greater number of post-culture T cells in response to CMV- than control antigen, but did not address the possible presence of IFN-γ secreting T cells in response to any non-CMV components of the antigen. To address this, control antigen-driven cultures could be analysed for IFN-γ secretion, compared to CMV antigen-driven cultures. Restimulating CMV antigen-driven cultures with unpulsed APC, as well as control and CMV antigens before the assay, would reveal any control antigen-induced IFN-γ secretion.

Taking these preliminary results, functional analyses of post-CMV antigen-driven T cell cultures from CMV seronegative donors, i.e. for the IFN-γ response or cytotoxicity assay remain important projects for the future.

Using our culture system in combination with other novel techniques, for example post-culture stimulation with anti-CD3 and anti-CD28 antibody coated beads [149], and/or inclusion of a reducing agent, e.g. NAC [155] to enhance antigen presentation may be pathways worth pursuing. The effects of other cytokines, e.g. IL-7 (T cell growth factor), or IFN-γ (induces expression of HLA-molecules), can be tested on their own or in combination.

The antigen employed in the generation of CMV-specific T cells in the culture system described here is a whole cell lysate derived from a human cell line infected with CMV. The clear advantage of such an antigen is that no prior knowledge of its constituents is required for the generation of a broad antigen-specific T cell response, making this a very simple and comprehensive approach. However, there are concerns about non-CMV specific T cell responses that may be induced by this antigen, as well as about the variable performance of different batches of CMV lysate, making standardisation impossible. T

cells specific for CMV antigens which cross-react with human antigens have been identified [156]. Whilst a culture system based around CMV lysate has great versatility, being applicable to all donor/patient populations, there are concerns about using such undefined antigens in clinical studies. The recently introduced changes in European clinical trials legislation may prohibit this approach for treatment of patients in the near future.

In the future, techniques for isolating/expanding CMV specific T cells for adoptive cellular therapy will have to depend on more clearly defined antigens, which can be produced under GMP conditions. In our hands, purified pp65, although identified as the most immunodominant protein of CMV, was quite ineffective at inducing a T cell response in our culture system when compared to CMV lysate (data not shown). Overlapping peptides spanning the whole of pp65 or other CMV proteins have shown great potential [62;63;132;140;157-159]. They are presented by APC on HLA molecules directly, not requiring intracellular processing. Combining overlapping peptides from several immunodominant proteins, such as pp65 and IE-1 may broaden the T cell response. Such peptide mixtures may be used to expand CMV specific T cells *in vitro*. Short stimulation with peptides also very effectively induces T cell IFN-γ secretion, facilitating identification and isolation of responding T cells.

HLA-tetramers or similar HLA-peptide polymers can very specifically isolate T cells against single peptides. Several groups have demonstrated the application of tetramers to isolate CMV-specific T cells from whole blood [160], PBMC [8;141;154;161;162], or from cultured T cells [48;49;57;58;62;71;134;143;163], and a first clinical study with tetramer-selected cells showed some success [47]. However, at the present, tetramer technology would only be applicable to donors/patients expressing the most common HLA-types. Even in these, major T cell responses may be missed completely, if restricted by other HLA alleles.

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Appendix

Chemicals and Consumables

Equipment	
Spectrophotometer	Amersham Biosciences
ABI 310 automated sequencer	Applied Biosystems
CEQ 8000 genetic analysis system	BeckmanCoulter
EPICS Elite flow cytometer	BeckmanCoulter
Automated Plate Harvester	Wallac
Scintillation sheet	Turku
Microbeta filter	Turku
Cytocentrifuge	Shandon
VarioMACS magnet	Miltenyi Biotech
LS/VS MACS column	Miltenyi Biotech
MS MACS columns	Miltenyi Biotech
Waterbath	Grant
Tissue Culture Plastics	
20μm pore-size syringe filters	Sartorius
5ml tube	BD Falcon
50ml tube	BD Falcon
15ml tube	BD Falcon
7ml bijou container	Sterilin
30ml universal container	Sterilin
Tissue culture flasks 25cm^2	Nunc
Tissue culture flasks 80cm^2	Sarstedt
Sterile round-bottom 96-well plates with lid	BD Falcon
1.8ml Cryovials	Nalgene
Cryocontainer: "Mr Frosty"	Nalgene
Assay Kits	
Interferon-γ Secretion assay	Miltenyi Biotech
Cell Labelling Reagents	
Mouse anti-human CD8-FITC	DakoCytomation
Mouse anti-human CD4-APC	BD
Mouse anti-human CD14-FITC	DakoCytomation
Mouse anti-human CD86-FITC	Serotec
Mouse anti-human CD83-FITC	Bioscience
Mouse anti-human CD40-PE	Serotec
Mouse anti-human HLAII-DP, DQ, DR	Serotec
HLA-tetrameric complexes	Proimmune
triCHROME (anti-CD3-PC5, anti-CD4-RD1, anti-CD8-FITC)	BeckmanCoulter
Anti-human CD14 microbeads	Miltenyi Biotech

Antigens	
CMV antigen	DadeBehring
Control antigen	DadeBehring
Staphylococcus Enterotoxin B	Sigma
Chemicals	
Chloroform	BDH
Isopropanol	BDH
Ethanol	BDH
EDTA	Sigma
Formamide	BDH
Sodium azide	Sigma
DMSO	Sigma
Molecular Biology Reagents	
Ultraspec RNA emulsion	BiotechX
hexanucleotide mix	Roche
Superscript Reverse Transcriptase	Invitrogen
hexanucleotide mix	Roche
RNAase Inhibitor	Roche
Geneamp PCR buffer	Applied Biosystems
magnesium chloride	Applied Biosystems
dNTPs	Bioline
Amplitaq DNA polymerase + GeneAmp buffer	Applied Biosystems
Oligonucleotide primers	BeckmanCoulter or Oswell
Glycogen	Ambion
Tamra-conjugated 500 basepair size standard	Applied Biosystems
performance optimized polymer 4	Applied Biosystems
CEQ 400bp size standard	BeckmannCoulter
CEQ buffer	BeckmannCoulter
Sterile water for injection	B Braun
Cytokines	
Recombinant human GM-CSF	Insight Biotech
Recombinant human CD40L	Alexis
Recombinant human IL-2	Insight Biotech
Recombinant human IL-4	Insight Biotech
Recombinant human IL-6	Insight Biotech
Recombinant human IL-15	Insight Biotech
Recombinant human IL-7	Insight Biotech
PGE_2	Sigma
Recombinant human TNF-α	Insight Biotech

Cell Culture	
X Vivo 20 culture medium	Biowhittaker
X Vivo 10 culture medium w/o phenol red	Biowhittaker
Dulbecco's PBS w/o Ca and Mg	Gibco
tritiated Thymidine	Amersham Biosciences
Ficoll	Pharmacia
BSA	Sigma
FCS	PAA Laboratories
Human serum albumin	Grifols
Computer Software	
Genescan 2.1 software	Applied Biosystems
Excel	Microsoft
Medcalc®	